Development of behavioural tasks for phenotyping of transgenic mice.

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Abstract

Many current models of cognition in mice focus on forms of memory. However, human neurodegenerative diseases present a much broader range of behavioural deficits that also require to be modelled. This thesis aims to describe the development of a battery of behavioural tasks that can assess attentional, mnemonic and circadian function in both normal and mutant mice. A novel mouse model of sustained attention using an analogy to the rat five choice serial reaction time (5CSRT) task is described. This task is of interest as attentional deficits are seen in patients with schizophrenia and Alzheimer's Disease. Transgenic mouse models of both of these diseases are currently under development.

Genetically-modified mice are typically generated on a mixed genetic background. Importantly, an extensive body of literature has described an extremely heterogeneous behavioural repertoire across the more commonly used strains. These strain differences could seriously influence the behavioural characterisation of any transgenic phenotype. This body of work includes a number of investigations of strain differences in some of the behavioural tasks used to phenotype transgenic mice.

Differences existed between two inbred mouse strains, C57Bl6/J and 129P2/Ola and one rat strain (Lister Hooded) on performance of the 5CSRT task. Between the mice the 129P2 strain was more competent at challenges that altered task predictability, whereas the C57 strain were better at low stimulus intensities. The rats were shown to be most perturbed by unpredictable stimuli presentations. The imposition of a response restraint challenge indicated that the rats were more impulsive than either mouse strain in this task.

Circadian behaviours were examined through analysis of timing and intensity of locomotor activity in the home cage under varying periods of strong light exposure. Mouse strain differences were seen for gross levels of activity and the time taken to entrain to a novel lighting schedule. Subsequent phenotyping of a $VPAC_2$ receptor knockout line, a novel mutant mouse generated on a mixed C57 and 129P2 genetic background, revealed a critical role for this receptor in the maintenance of normal circadian rhythmicity.

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Declaration

I declare that the work submitted here is composed by this candidate and that appropriate acknowledgement has been given where reference is made to the work of others.

Signed:

Date: 14/3/03

"While I'm still confused and uncertain, its on a much higher plane, d'you see, and at least I know I'm bewildered about the really fundamental and important facts of the universe."

Treatle nodded. "I hadn't looked at it like that", he said, "But you're absolutely right. He's really pushed back the boundaries of ignorance"

From "Equal Rites" by Terry Pratchett

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Publications arising from this thesis

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Table of contents Page

List of Figures, Tables and Equations Page

1 Introduction

1.1 Human to animal and back

Treatment of human diseases and disorders requires understanding of the mechanisms behind the perturbation. Once a mechanism of disruption is identified it becomes possible to suggest potential methodologies to restore normal function. Within the central nervous system it is unlikely that any single disease exists as a simple one step mechanism causing the pathological condition. Diseases of the CNS generally present with multiple symptoms that result from a complex set of pathologies. For example Alzheimer's Disease patients exhibit various structural abnormalities within the CNS post-mortem including neurofibrillary tangles and amyloid plaques. Phenotypic outcomes of a disease can be related to either unitary pathologies or the interaction of multiple pathologies. In the search for treatments to human diseases it has become necessary to model aspects of human pathology *in vitro* and *in vivo*. Furthermore, whilst it is possible to ask a human patient how they are feeling or what they are seeing during a test battery it is impossible to ask a rat or primate. We are limited to merely observing non-human subjects and can only judge them on their responses.

Many schizophrenic patients suffer attentional deficits that are revealed through tasks such as the span of attention task and continuous performance task (Ito, Kanno et al., 1997;Addington and Addington, 1997;Addington and Addington, 1998;Oades, Rao et al., 2000;Nieoullon, 2002). It has been suggested that this may result from impaired sensory gating (Nieoullon, 2002) leading to inappropriate filtering of relevant environmental stimuli. Studies have shown that many schizophrenic patients show abnormal frontal lobe structure (Andreasen, Swayze et al., 1995;Baare, Hulshoff Pol et al., 1999) with reduced grey matter volumes in some frontal areas. Furthermore, a reduction in frontal region activation has been seen in schizophrenic patients during task performance as reviewed by Weinberger (Weinberger, Egan et al., 2001). However this reduced activation may be related to the type of task the subject is doing (Curtis, Bullmore et al., 1998;Curtis, Bullmore et al., 1999). Patients who present with frontal lobe damage (commonly through car accidents) have also been shown to exhibit attentional deficits (personal communication). These patients sometimes latterly present with schizophrenic symptoms. This potentially links a physical condition and an observable phenotype with a psychiatric disorder. Therefore frontal lobe damage resulting in attentional impairment may suggest a useful animal model of

schizophrenia. Hence it is of interest to develop an attentional task that is sensitive to frontal perturbations in order to examine potential treatments that may restore attentional functioning.

Over the years a comprehensive body of literature has been built regarding the development of tasks that test memory and attention in primates and latterly rats. An important consideration in developing many of these tasks was to attempt to demonstrate 'behavioural homology' between humans and the test animals (Roberts, 1996). These tests include analogies of existing human tasks such as the Wisconsin Card Sort Test in primates (Roberts, De Salvia et al., 1994;Dias, Robbins et al., 1996). These analogous tasks still used the shape and colour dimensions and touch screen technology thus allowing close comparison between human and non-human primates. Recently the attentional set-shifting basis behind the Wisconsin Card Sort Test was developed into a rat task (Birrell and Brown, 2000). The rules remained similar; a trained subject was presented with pairs of two dimensional stimuli but only one dimension was important at a time. Instead of learning to attend to shapes rather than colours (or visa versa) like the human and monkey the rats were required to differentiate smells and textures. In this way they were accessing potentially more salient sensory modalities. Colacicco et al. have recently described a mouse variant of the rat set shifting task (Colacicco, Welzl et al., 2002) which also utilised the smell-texture axis.

Following the human indications of prefrontal cortex perturbations affecting cognitive performance the effects of selective lesions within the frontal cortex circuitry in rats and non-human primates was subsequently studied (Muir, Everitt et al., 1994;Wilkinson, Dias et al., 1997;Collins, Roberts et al., 1998;Chudasama and Muir, 2001). These studies have shown that selective lesions can result in a variety of impairments in performance such as rate of responding, accuracy of response or extent of neglected stimuli. Following the localisation of behavioural impairments to specific nuclei within the brain it proved possible to infer a role of specific receptors in mediating the impairment. This suggests possible pharmacological manipulations that might result in either the behavioural impairment or its amelioration.

A more recent approach to testing a pharmacological hypothesis is to genetically mutate the target receptor and measure any change in function. Most common is to knock out the receptor and define the phenotype resulting from its absence. In mammals this is most easily done in the mouse for reasons some of which shall be discussed later. Therefore one

approach to looking at means of improving frontal function in humans is to develop a mouse task that is sensitive to frontal perturbations.

1.2 Visual attention

Visual attention was originally tested in enlisted men using a test called Leonard's 5-choice test of serial reaction (cited in Wilkinson 1963). In this task the subjects were required to attend to an array of five light bulbs and a response at the associated contact would extinguish an active light and initiate the activation of a new stimulus. The initial studies investigated the effects of stressors such as sleep deprivation and constant white noise on performance (Wilkinson, 1963) with and without performance feedback. Latterly more sophisticated tasks such as the Cambridge Neuropsychological Test Automated Battery (CANTAB) were developed which comprises a selection of visually-mediated tasks some of which are sensitive to frontal lobe dysfunction (Roberts and Sahakian, 1993;Fray and Robbins, 1996;Robbins, James et al., 1998). These tests have revealed deficits in a wide range of patient groups such as Alzheimer's Disease (Sahakian, Owen et al., 1993;Dorion, Sarazin et al., 2002) Attention-Deficit Hyperactivity Disorder, depression (Shah, O'Carroll et al., 1999), Huntington's Disease, bipolar disorder (Sweeney, Kmiec et al., 2000;Harmer, Clark et al., 2002) and schizophrenia (Elliott, McKenna et al., 1995;Pantelis, Barnes et al., 1997). In some cases the tests developed in the CANTAB battery were derived from existing primate tasks and have subsequently been transferred to the clinic.

Following the detection of performance deficits in visual attention tasks by humans the tasks were adapted for work in primates and rats as noted previously. This allowed investigation of the neurotransmitter pathways involved in attention (reviewed by Bushnell (Bushnell, 1998)). In the rat, one of the well-established analogies to the 5-choice test of serial reaction was designed by Carli et al (Carli, Robbins et al., 1983). In this task the rats were trained to respond to brief light bursts at one of five random locations in order to receive food reward. In this example a human task was transferred almost entirely intact into testing with rats, rather than the set-shifting paradigm described earlier that moved to a different set of modalities (Birrell and Brown, 2000). In the 5SCRT task, usually following training, the rats could then be challenged through either manipulation of the task parameters or through pharmacological impairments (Jones and Higgins, 1995) or lesions (Robbins, Muir et al., 1993;Muir, Everitt et al., 1996). The benefits of these approaches include the possibility for acute and highly localised damage and the ability to use the same animals as controls (pre vs. post) surgery (Robbins, Muir, Killcross, and Pretsell, 1993). In rats, chemical and surgical

lesion studies have demonstrated the effects of noradrenaline, serotonin, acetylcholine and dopamine pathways on performance of attentional tasks to varying degrees. However these invasive approaches are not readily amenable to developmental assessment, lesion and surgical approaches tend to utilise adults not neonates. Therefore development of a mouse variant of the rat 5CSRT task might prove useful in investigating genetic as well as structural aspects of frontally-dependent behavioural performance.

As with any new task many aspects of the resultant behaviour must be analysed, it is important to demonstrate that as many component behaviours as possible are functioning normally. For instance the 5CSRT task involves a high degree of locomotor activity and a dependence on adequate visual sensitivity. Impairments in performing the 5CSRT task could be due to hypoactivity, a subject with difficulty moving might be unable to reach the stimuli within an appropriate length of time resulting in either poor or slow performance. Due to the emphasis on vision in the rat task, any impairments could also be due to visual problems. Deficits in acquisition could be due to cognitive impairments impeding procedural learning or recall. As an appetitive task the motivational state of the subject would also be paramount in their ability to perform adequately. Therefore as well as the primary goal of developing a mouse analogy to the rat 5CSRT task it is important to demonstrate that as many subservient systems as possible are functioning normally. Only once the new task is optimised for the normal population does it become possible to impose challenges on the subjects through lesion, pharmacological or genetic manipulation. Therefore, it was decided to develop a battery of tests to investigate various aspects of cognition and physiology in the mouse. As well as the 5CSRT task, it was important to look at general locomotor activity under comparable conditions to those anticipated to be used in the 5CSRT task. Also vision could be assessed through devising a circadian behavioural task, which would involve both locomotor and visual components. Procedural learning and memory could be assessed through development of other cognitive tasks that use separate cognitive processes previously demonstrated in mice. To this end it was decided to develop a delayed non-matching to position task. This kind of task involves components of procedural learning ad recall, appetitive responding, as well as working memory (Dunnett, 1993).

1.3 DNMTP

A delayed matching or non-matching to position task is a discrete trial delayed response task (Dunnett, 1993). The basic operant task involves two or more possible response locations and the subject must determine which location to respond to based upon prior exposure to a

cue. Each trial is independent, and the trial difficulty is defined by the delay between cue presentation and the opportunity to respond, hence varying the amount of time the cue location information must be retained for. These prove very effective tasks for measuring working memory in primates and rats Most operant variants of this task are appetitively rewarded, this usually requires the subject to be given a sought-after food reward. Therefore successful execution of a D(N)MTP task requires adequate motivation to perform. Since the premise of a matching or non matching to position task is merely to train a subject to discriminate between two locations over an increasing memory load then other forms of motivation might be used such as the impetus to escape a water maze or to avoid foot shock. Until recently there has been little published work on mouse variants of the operant task, delayed non-matching tasks instead using various maze paradigms. The literature includes mouse versions of non-matching to place radial arm maze (Marighetto, Micheau et al., 1993) and T-maze tasks (Cho and Jaffard, 1994;Beracochea and Jaffard, 1995;Ward, Billinton et al., 2001) as well as water mazes (Means and Fernandez, 1992).

1.4 Circadian biology

Circadian mechanisms are important in many species for seasonal behaviour – breeding, seasonal dimorphism for camouflage, feeding patterns, etc. Circadian rhythms are oscillations in an organism's behaviour that cycle with a repeating unit of approximately 24 hours. Complex biochemical clocks integrate a variety of environmental stimuli, one of the most predominant of which is light, to adjust an organism's internal state to the local day or night. In isolation studies with humans, studies in which the subject was commonly placed in underground chambers or caves, the external environmental cues such as daylight can be excluded. Under such conditions most humans continue to adopt a repeating pattern of behaviour – sleeping, waking, hunger and activity but with a repeating period of approximately 25 ± 0.5 hours (Aschoff, 1984). Furthermore, once environmental time-givers (zeitgebers) had been excluded the experimenter could resynchronise the subjects to a totally new daily cycle by altering the timing of light presentations. The process of adjusting internal rhythms in response to external stimuli in this way is called entrainment. In much the same way the circadian clock of smaller mammals can be tested by measuring the timing of activity in their home cage. In mammalian chronobiology, wheel running is often taken as a measure of circadian behaviour and by inference the central regulation of circadian rhythms.

The mammalian internal clock co-ordinates rhythmic patterns such as hormonal secretion, temperature fluctuations and reproductive, locomotor and feeding behaviours (Hastings, 1995;Sassone-Corsi, 1998). By convention the clock must have three components, firstly an input pathway to link external environment to internal synchronisers. Secondly comes the central oscillator and finally there are the output pathways that express repeating rhythms.

In mammals the central oscillators lie within the suprachiasmatic nuclei (SCN). The definitive work that demonstrated the absolute requirement for the SCN in co-ordinating internal rhythms to the environment came in 1987 where rhythmic activity was restored in arrhythmic, SCN-lesioned hamsters by grafting of foetal SCN tissue into the brain (Lehman, Silver et al., 1987). Later work demonstrated that restored rhythms are based upon the circadian period (duration of cycle) of the donor subject, not the recipient, i.e. the circadian period of the animal was in some way defined by the SCN tissue it possesses. Furthermore, Silver et al showed that the signal between transplanted tissue and recipient was a diffusible signal by isolating the transplant tissue inside a semi-permeable capsule and still obtaining restoration of a 24 hour period (Silver, LeSauter et al., 1996). The principal input to the SCN is the retinohypothalamic tract (RHT), which in turn receives input from the retinal photoreceptor cells. In this way the SCN receives photic entrainment cues. Non-photic stimuli to the SCN such as handling and injection arousal are more dependent upon the geniculohypothalamic tract (GHT) reviewed in Hastings 1997 (Hastings, Duffield et al., 1997). The SCN release numerous peptides directly including vasopressin and indirectly innervates the pineal gland which has no direct response to photic stimuli. The pineal gland expresses a group of proteins essential for the circadian release of melatonin – a hormonal hallmark of night. However, differences have been reported for various mouse strains with regards to melatonin metabolism (Kopp, Vogel et al., 1998). Following the identification of light-inducible genes in the SCN (Morris, Viswanathan et al., 1998) and the isolation of several mammalian clock genes with circadian expression (Tei, Okamura et al., 1997) or which encode proteins essential for normal circadian activity (King, Zhao et al., 1997) there has been renewed interest into the genetics behind circadian behaviour.

This interest in the genetics behind circadian rhythmicity has revealed a complex series of interacting molecular loops that drives the recurring rhythms in the RNA and subsequent protein levels within individual cells. The molecular clock is driven by two transcription factors, CLOCK and BMAL1, within the nucleus. These transcription factors heterodimerise and can then activate the transcription of a series of *period* and *cryptochrome* genes. The

resultant mPER and mCRY proteins then translocate back into the nucleus to exert feedback effects on the CLOCK BMAL1 heterodimer. The mCRY proteins act on the heterodimer to inhibit transcription of *mPer* and *mCry* genes thereby creating a negative feedback loop. A positive feedback loop appears to be regulated through an orphan nuclear receptor gene. The transcription of the *Rev-Erbα* gene results in a protein that appears to repress *Bmal1* transcription (Ueda, Chen et al., 2002;Preitner, Damiola et al., 2002). The transcription of the *Rev-Erbα* gene is activated by the CLOCK/BMAL1 heterodimer, therefore the entry of the mCRY proteins into the nucleus to inhibit the action of the CLOCK/BMAL1 heterodimer conversely inactivates the transcription of *Rev-Erbα* gene. With the now inactive *Rev-Erbα* gene the transcription if *bmal1* is once again activated. The timing of these positive and negative feedback loops creates an output signal that results in the circadian phenotype. The importance of these proteins to the eventual circadian phenotype is clearly demonstrated by double knockout mice for either the *cryptochrome* or *period* genes, which demonstrate a complete arrhythmia in constant darkness (van der Horst, Muijtjens et al., 1999;Zheng, Albrecht et al., 2001).

1.5 Gene targeting in mice

Molecular biology and gene-targeting techniques in particular have developed to the point where it is possible to create highly specific alterations to single genes in mouse strains. These mutants are then studied for biochemical, morphological and behavioural alterations from the 'norm' as defined by wild-type (wt) mice (Silva, Simpson et al., 1997). Most gene targeting itself typically uses embryonic stem (ES) cells commonly derived from Strain 129 mice. The phenomenon of homologous recombination, by which two separate genomic sequences can exchange regions held within flanking sequences of extensive homology, permits targeted mutation. The DNA sequence of interest is modified, usually either to alter eventual protein translation or function. The modified gene is then inserted into a genetic construct between two specifically designed flanking sequences. These flanking regions are homologous to regions flanking the eventual target gene. Thus when the genetic construct is introduced into the ES cells containing the target gene there is a possibility that the target gene will be exchanged with the modified gene by the homologous recombination event. By the use of selection factors only the ES cells incorporating the modified gene in the correct part of the ES cell genome will survive (Gerlai, 1996c;Holsboer, 1997). Any correctly modified ES cells are then injected into a mouse blastocyst, which is in turn implanted into a pseudo-pregnant female. This creates chimeric mice in which some cells, descended from

the modified ES cell, contain the altered genome. If there is transmission of this altered genome through the germline by mating chimerae to standard inbred mice the offspring are heterozygous for the alteration. Interbreeding of this heterozygous F1 generation allows the establishment of the modification into a mutant line. Such heterozygous crosses create an F2 generation comprising of wild-type, heterozygous and homozygous mutant offspring at the targeted allele.

Since the early 1990's the field of targeted mutagenesis in mammals, predominantly in mice, has been rapidly expanding. Many of these animals have been developed for determining the functions of recently identified genes. Frequently these mutations target proteins found within the brain and as such assessing the behavioural phenotype of such mutants is vitally important in attempting to deduce the proteins' role. Animal behaviourists need to understand the various techniques available to the molecular geneticist so that interpretation of phenotypes takes into account the limitations and genetic permutations the techniques will create.

1.6 Genetic manipulation approaches

Genetic manipulation can be comprehensive through either addition or deletion of an entire gene or partial by modifications to part of the gene to alter its function. It has allowed us to investigate the complex molecular and cellular pathways underlying behaviours related to social interaction, learning, memory, motor co-ordination and control (Silva, Simpson, Takahashi, Lipp, Nakanishi, Wehner, Giese, Tully, Abel, Chapman, and et.al., 1997). Genetic manipulation has significant appeal in that the manipulation is often precise and can potentially remove a gene product without the side effects and limitations of drugs such as drug sequestering, toxicology, tissue permeability, trafficking. However, genetic manipulation is very expensive and time consuming during the process of generating a stable modification and it is difficult to prove that any effect is directly due to the modification. Furthermore, once the mutation is stably expressed it may result in embryonic or early postnatal death negating further behavioural analysis.

1.6.1 Additive manipulations

The most common additive manipulation is pronucleus insertion transgenesis. This process involves injection of DNA into the male pronucleus of fertilised egg prior to implantation. The DNA is incorporated into the male pronucleus and subsequently becomes part of the

genome of the resultant zygote. Selective breeding of the transgenic offspring can establish the newly integrated gene in the genomic sequence and allow maintenance of the mutant line. This process is used repeatedly to insert multiple copies of a gene allowing overexpression of the encoded protein (Seabrook and Rosahl, 1999). However this technique can be unpredictable both in the site of genomic integration and level of newly integrated gene expression (Holsboer, 1997). Since the injected DNA will integrate at a random point in the genome it can conceivably disrupt other gene functions by interrupting promotor or exon coding sequences. For this reason it is inadvisable to generate homozygous mutants by mating heterozygotes from within a single line as any perturbation to other genes will now be expressed on both alleles in the homozygous mutants. This would create a homozygous knockout for an unrelated and possibly undefined gene. This obviously aggravates phenotyping difficulties.

Recent developments in molecular genetics have generated solutions to some of the more wide-ranging problems of genetic manipulation thus far described.

1.6.2 Antisense mRNA

A subtle modification of pronuclear insertion genetics involves the insertion of antisense RNA. This is designed to bind to the mRNA encoding the target protein essentially blocking all post-transcriptional events such as protein translation from the gene by ribosomes. Thus the gene's normal functioning is impaired whilst the gene itself is left intact (Holsboer, 1997). This method was first used to inhibit gene expression in transgenic animals in 1988 where an antisense RNA construct was injected into fertilised mouse eggs heterozygous for the shiverer mutation (shi/+). The construct caused a reduction in myelin basic protein in the mice creating a phenotype comparable to the homozygous shiverer mutants (shi/shi) (Katsuki, Sato et al., 1988). The method suffers from the same potential problems previously mentioned for the pronuclear insertion technique in that the site and extent of construct expression is random. However it does demonstrate how the pronuclear insertion technique can be used to inhibit gene function as well as overexpress it.

1.6.3 Site specific mutagenesis

Site specificity in gene targeting relies on tissue-specific promotors. One method involves creating a genetic construct in which Cre recombinase expression is controlled by, e.g. a

neuronal-specific promotor, and expressing this construct in a mouse. The Cre-expressing mouse is mated with another mouse that expresses the neuronal target gene flanked by loxP recognition sites. In any resultant double construct-expressing animals, the target gene would only be excised in those tissues in which Cre recombinase is synthesised under the control of the tissue-specific promotor. (Gingrich and Roder, 1998). Thus, we have the ability to study the effects of gene alteration or knockout in specific tissues as opposed to the whole body.

It is also theoretically possible to execute compensation studies where one gene is removed and another gene from the same family is inserted (all under tissue specificity) to determine whether genes can substitute for each other (Holboer 1997).

1.6.4 Inducible gene regulation

Possibly one of the most exciting developments in recent mammalian molecular genetics has been the establishment of time-sensitive gene regulation techniques. This provides an approach to avoid the potential of compensatory mechanisms previously theorised to develop following neonatal genetic manipulation. It usually requires attachment of an inducible promotor to the target gene prior to integration into the genome. This allows expression of the modified gene upon either endogenous or exogenous application of the inducer. Thus, the modified gene and subsequent gene products shouldn't influence the natural development of the organism until it is activated or 'induced' (Gerlai, 1996c;Holsboer, 1997;Gingrich and Roder, 1998). In this way it may be possible to prevent compensatory or substitution mechanisms developing. Furthermore the individual animals act as their own controls since one can run them in paradigms both before and after modified gene expression.

A recent example of this approach was a so-called "rescue" control where functional *fyn* gene expression was restored in the mutant mouse (Kojima, Wang et al., 1997). The authors attempted to address which behavioural and electrophysiological deficits seen in *fyn*-deficient mice were most closely linked to *fyn* expression. A genetic construct placed *fyn* cDNA expression under the control of a CaMKIIα promotor. This was chosen so that the promotor should only be active in CaMKII-expressing cells thereby restricting activity to postnatal forebrain neurons (Gingrich and Roder, 1998). This example demonstrates that careful selection of the promotor sequence can create both tissue and temporal specificity. When this *fyn* overexpressor line was mated with a *fyn* KO line a new mutant line was created. In this new line, *fyn* expression was absent in the neonate but was present several

weeks after birth. They reported morphological defects to the hippocampus comparable to those seen in other *fyn*-deficient mice but reported a restoration or rescue of LTP to wt levels in the adult. Whilst this isn't conclusive proof of the role of *fyn* protein, it does imply a requirement of the *fyn* protein for normal LTP function (Kojima, Wang, Mansuy, Grant, Mayford, and Kandel, 1997;Lipp and Wolfer, 1998). To date there have been no reported findings of rescued behaviour in these animals.

However, the Kojima paper hasn't addressed the problem of *fyn* transgene integration. They used the pronuclear injection method thus can't be certain that additional genes weren't interrupted. The mutant line containing the CaMKIIα promotor-linked *fyn* gene also contained endogenously active *fyn* proteins which could have been transferred into the final mutant line resulting in limited *fyn* gene expression throughout development. Of more concern is the finding that the LTP deficit previously reported (Grant, O'Dell et al., 1992) in *fyn*-deficient mice wasn't recorded until the mice were 10 weeks old. Younger than 10 weeks and the *fyn*-deficient mice had normal LTP patterning. However, the paper does prove that the hippocampal structural abnormalities aren't the direct cause of LTP impairment since the impairment was absence in young mice.

The most widely used method of targeted genetic manipulation utilises a process called homologous recombination In this process, two separate genomic sequences containing regions of extensive homology may exchange the sequence held between homologous regions. A genetic construct; generally the DNA sequence encoding a modified gene of interest; is introduced into embryonic stem (ES) cells extracted from the inner cell mass (ICM) of mouse blastocysts. Some of the ES cells take up the DNA construct and then incorporate it into their own genomic sequence by homologous recombination. Through the use of several selective factors only the ES cells containing the modified gene in the correct part of the ES cell genome will survive. These modified ES cells are injected back into mouse blastocysts, which are then implanted into a pseudopregnant female (a female mated with a vasectomised male so that she is hormonally receptive for blastocyst implantation). ES cells have the capacity to become any of the cell types of the developing mouse. Thus, in the developing embryo, some cells will possess the normal wild-type (wt) genotype and others, descended from the modified ES cell lines, will express the mutant genotype. If some of the germ line cells are descended from the modified ES cells then the mutation can be transmitted into the genome of subsequent generations. Breeding such chimeric mice with wt mice will produce a limited number of animals containing one copy of the wt gene (from the

wt parent) and one copy of the modified gene from the chimeric parent. These F1 heterozygotes can be bred together to produce an F2 generation containing wt, heterozygous and homozygous mutant offspring. The effect of the mutation is then typically assessed using these three F2 groups. (Miner, 1997;Holsboer, 1997). Unfortunately, this system nominally generates homozygous mutants that do not express the unmodified target gene anywhere. Furthermore, the F2 generation possesses a combination of genes derived from both the ES cell and blastocyst donor strains. This creates a variable recombinant genotype within the litters, which may mask weak phenotypic changes and prevents the use of wt littermates as appropriate genetic controls (Gerlai, 1996b;Silva, Simpson, Takahashi, Lipp, Nakanishi, Wehner, Giese, Tully, Abel, Chapman, and et.al., 1997). This is particularly problematic because, until recently, the ES cell donor and blastocyst donor strains were different - commonly 129 substrains supply the ES cells and C57Bl/6 and BALB/c substrains supply the blastocysts and host mothers.

Use of this ES cell-based system has allowed creation of several significant genetic modifications. The 'null mutation' involves the insertion of a random sequence of DNA into the gene of interest within the ES cell thus rendering the gene non-functional in the resultant chimeric animals (Gerlai, 1996b). A target gene can be removed from the chromosome by developing a construct that incorporates the flanking regions but has the target gene absent. These sorts of constructs generate the knockout (KO) mice. Another approach to removing a DNA sequence utilises the Cre recombinase enzyme from bacteriophage P1. This enzyme recognises and cuts at certain DNA sequences called loxP sites. By flanking an unwanted piece of DNA with loxP sequences and introducing the Cre recombinase-expressing gene, the loxP flanked (floxed) gene is neatly removed (Holsboer, 1997;Gingrich and Roder, 1998). This approach was used by the first reported targeted gene deletion in 1995 where Cre recombinase deleted a 'floxed' segment of the DNA polymerase B gene in various tissues throughout a mutant mouse strain (Kuhn, Schwenk et al., 1995).

In mammals these techniques are most accessible when working with mice. This popularity is in part due to robust embryonic stem cell lines, a short gestation cycle and comparatively cheap husbandry costs in terms of space and food.

To date targeted transgenesis has been limited by technical difficulties to often using ES cells that are easily derived, such as those derived from Strain 129 mice and blastocysts are commonly from C57Bl/6J mice. This results in chimeric mice containing both C57Bl/6J and

S129 genomic sequences. Furthermore, the crossbreeding of the chimerae to a standard inbred mouse line results in offspring with a yet further complicated genetic background. The recombinant genetic background generated by crossbreeding of two separate strains has in recent years been considered a major potential confound to the study of gene function in this way (Crusio, 1996;Lathe, 1996;Gerlai, 1996a;Gerlai, 1996c;Silva, Simpson, Takahashi, Lipp, Nakanishi, Wehner, Giese, Tully, Abel, Chapman, and et.al., 1997). Many have reported strain-dependant differences between inbred mouse strains to a wide variety of paradigms (Crawley, 1996;Crawley, Belknap et al., 1997;Owen, Logue et al., 1997;Paylor and Crawley, 1997;Wolfer, Muller et al., 1997;Logue, Owen et al., 1997;Kelly, Rubinstein et al., 1998;Crabbe, Wahlsten et al., 1999) to name but a few. In many cases the concern is that alleles which influence the inter-strain variability can randomly segregate at polymorphic loci in the F2 generation of a two-strain hybrid. Thus, even though the littermates of genetically-modified mice are generally taken as the 'appropriate' wt control, the genetic variability within the litter is such that they are far from identical – each animal contains a variable combination of alleles from the parental strains. This problem is somewhat alleviated by multiple backcrossing into a preferred strain. However, it is argued that some alleles closely associated to the original modified gene will always be transmitted to the next generation alongside the modified gene (Lathe, 1996;Gerlai, 1996b;Gerlai, 1996c). The strain a mutation is expressed in can also have an influence on the expression of a modified gene as seen by a strain-dependent attenuation of the lethality following disruption of the EGFR (epidermal growth factor receptor) gene (Sibilia and Wagner, 1995;Threadgill, Dlugosz et al., 1995). The 129 substrains, from which ES cell lines are most easily derived, have been reported to have a high degree of genetic variability especially between ES cell lines and their presumed parental substrain (Simpson, Linder et al., 1997). Some of the early circadian work (not presented here) utilised University-maintained 129/Ola stock. A second iteration used commercially available 129P2/Ola mice to investigate the possibility that segregation within the University stock was sufficient to significantly influence the animals' response to changing light and dark stimuli. Precedents for unusual genotypes linked to a single source do seem to exist, recently the C57Bl/6J mouse was reported to possess a deletion of the α-synuclein locus only in mice supplied by Harlan UK (Specht and Schoepfer, 2001). Closely-related populations (C57Bl/6) from the same source and other suppliers (C57Bl/6N, Charles River UK) did not possess this deletion. Perhaps more convincing is the failure to detect this deletion in C57Bl/6J genomic DNA from Jackson Laboratories, USA. However, these mice were unimpaired in a hippocampal-dependent spatial learning task (Chen, Specht et al., 2002).

The inbred mouse strains predominantly used in murine research often express homozygous deleterious alleles which cause phenotypic abnormalities. As such, most inbred mouse strains possess endogenous deficits in specific areas such as hippocampal-dependent learning (DBA and 129 strains), visual acuity (BALB/c and C3H) and response to limited audible stimuli (C57Bl/6) (Gerlai, 1996b;Silva, Simpson, Takahashi, Lipp, Nakanishi, Wehner, Giese, Tully, Abel, Chapman, and et.al., 1997). This may limit the usefulness of any particular strain for specific paradigms, e.g. with a gradually declining auditory response to certain frequencies C57Bl/6 mice might be disadvantageous in an extended duration startle response paradigm. However, if the experimenter's interest lies in gain of function then a depressed basal performance could be useful in avoiding potential ceiling effects (Gerlai, 2001).

Whereas individual inbred strains have deficits in certain areas of phenotypic analysis, the generation of F1 hybrid strains from the mating together of two separate strains will remove the effects of any homozygous deleterious alleles by making offspring that are heterozygous for all alleles thereby removing the deficits seen in pure inbred strains. The resultant phenotype might be considered to be a combination of additive and dominant genetic effects that were anticipated to generate a performance ranging between the limits of the parental performances. However, several hybrid strains have been reported as showing better performance than either of their parental strains in the Morris Water Maze (Owen, Logue, Rasmussen, and Wehner, 1997) and locomotor activity (Logue, Owen, Rasmussen, and Wehner, 1997) through a process called Hybrid Vigour (Wolfer, Crusio et al., 2002). Miner's work supports the theory that the phenotype of the F1 generation, in this case descended from a C57Bl/6 x 129/SvJ mating, doesn't always reflect additive effects and the dominant alleles of the parental strains. The F1 generation was indistinguishable from the C57Bl/6 parental strain when looking at short term habituation to a novel environment and spontaneous activity levels. In contrast, only the F1 mice showed a reduction in activity when four exposures to the test environment were spread over a two week period (Miner, 1997). Thus, characterisation of the phenotypic responses of the parental strains doesn't necessarily define the full range of outcomes possible from their offspring, with enhanced performance in the F1 hybrid line being possible. This process has led numerous groups to use F1 lines as their baseline phenotype onto which genetic manipulations are imposed. For instance some pharmaceutical companies backcross all their knockout mice onto a C57Bl/6:129SvEv F1 line as standard (Collinson, Kuenzi et al., 2002).

For a wide variety of currently-used paradigms many of the commonly used inbred mouse strains have been tested and patterns of strain response have been noted. However, work using ethanol to look at genetic factors in drug-related behaviour has shown that these patterns are unpredictable. Some strains of mice are sensitive to certain ethanol response parameters and insensitive to others. Furthermore, the pattern of strain and substrain sensitivity is dose-related (Crawley and Paylor, 1997). These differences may partially be explained by strain variations in drug handling (uptake, transport and metabolism) through which comparable drug administrations may not result in comparable brain or tissue concentrations. Here pharmacokinetics impinges on true drug sensitivity further confounding the investigator.

As molecular genetics techniques have improved, so several murine models of human cognitive disorders such as Alzheimers Disease (reviewed by Seabrook 1999) have been created as well as many receptor subunit dysfunction lines (Miner, 1997). Thus behaviourists must begin to develop or adapt novel paradigms to assess not only acute and chronic perturbations to phenotype such as may be required of drug studies but also develop methods for assessing progressive alterations to phenotype such as in models of Alzheimer's Disease. These longitudinal studies are currently being carried out through the development of acquisition and performance maintenance protocols in this lab and many others around the world.

1.7 Aims of the thesis:

By developing a series of interlinking tasks that cover various aspects of behaviour it was hoped that it would be possible to address divergent aspects of the cognitive and physiological condition seen within human patients. For instance, elderly depressed patients present with frontal lobe-sensitive cognitive deficits (Beats, Sahakian et al., 1996) but also possess disturbed sleep-wake cycles as reviewed by Bunney et al. (Bunney and Bunney, 2000). Therefore developing circadian paradigms as well as models sensitive to frontal perturbations would prove useful in understanding this disorder. To this end I have attempted to establish a series of independent tasks to address various aspects of cognitive control.

I wanted to establish murine models derived from previously established rat paradigms capable of characterising functions such as visual attention, procedural learning, perseverative responding and short-term memory. I also wanted to validate these tasks where possible with rats.

On the strength of the literature regarding the vast differences between various inbred mouse strains I wanted to investigate the behavioural differences within mouse strains used in this laboratory for both behavioural and transgenic studies.

As well as cognitive tasks it was decided to investigate innate circadian behaviours because some neurodegenerative disorders exhibit circadian deficits. It was decided to establish a paradigm that was used in both inbred mouse strains and a novel circadian mutant.

2 General Materials and Methods

This Chapter describes the common features of many of the experiments described within this thesis. Any major departures from these basic features are noted in the relevant portion of the text.

2.1 Animals

2.1.1 Animals

All animals used in this work were adult male rodents. Adult C57Black/6J (C57Bl/6J, generally abbreviated here to C57) and CBA/CaCrlBR (abbreviated to CBA here) mice were purchased from Charles River UK Ltd, Margate, UK at either a starting weight of 17 to 25 g or aged of 4 to 6 weeks old. Mice ordered by age were of comparable weight to weight-ordered mice. Adult 129P2/OlaHsd (abbreviated to 129P2 according to guidelines on nomenclature of strain 129 mice (Festing, Simpson et al., 1999) on the Jackson Laboratory website (Blake, Richardson et al., 2002) mice were ordered from Harlan UK Ltd, Bicester, UK at a starting weight of 20 to 25 g. Male Lister-Hooded Rats (abbreviated to LH rats) were also ordered from Charles River UK Ltd. at an start weight of 220 to 240g. Additional groups of animals are described in the relevant sections that follow.

2.1.2 Housing

All animals not involved in circadian studies were initially group housed (4 mice per cage, 3 rats per cage) with free access to tap water and food (RM1, low protein rodent maintenance diet, SDS Ltd, Essex, UK). Mice were housed in clear polycarbonate cages (365 (long) x 207 (wide) x 140 mm (high) with floor area of 530 cm^2, Tecniplast, Kettering, UK) with wood chip bedding. Rats were housed in clear polycarbonate cages (480 (l) x 265 (w) x 210 mm (h), floor area 940 cm^2, Techniplast) with a mixture of wood chip and wood shavings. All cages had a wire grid lid allowing for air circulation and roof running. Cages were stored in open metal racks in close proximity to neighbouring cages. No effort was taken to prevent subjects viewing the cages in their immediate vicinity. Both housing and experimental rooms are air-conditioned (21 ± 2 °C) and under a 12 hour light 12 hour dark cycle (12:12 LD) with lights on at 07.00 BST. Adjustment for daylight saving was not used in the animal facility.

Behavioural testing was completed during the light half of the LD cycle unless otherwise noted.

2.1.3 Food restriction protocol

A food restriction protocol was used in most of the behavioural studies; exceptions to this will be noted in the relevant chapters. The standard protocol required the animals to be given at least a minimum of 3 days but ideally one week and *ad. libitum* feeding upon arrival during which time they were earmarked for later identification. After the *ad. lib.* feeding period the subjects were weighed in the morning to establish the free-feeding weight. A target weight of 85% of the free-feeding weight was calculated and subsequent food was titred to attain the subjects target weight. Subjects were weighed daily in the morning of weekdays and feeding always occurred post-testing, usually in the afternoon. Feeding on non-test days occurred earlier so an additional 0.5 g per mouse was given to account for the increased delay between feeding and testing. Food restriction was then maintained throughout the behavioural study unless otherwise noted. To allow for growth the target weight was slowly increased by approximately 2% per week unless it appeared to impede acquisition of the task at hand. Occasionally a break in a study was necessary, during this time subjects were placed back on *ad. lib.* feeding and when testing was restarted their current *ad. lib.* feeding weight was taken as the baseline and a new target weight calculated. In such cases further accommodation for potential growth was not taken into account. This was the standard protocol but it was subject to change if animals were too light (approximately 17g starting weight) or if subjects became ill during testing. In the case of very light animals weight was not taken down below 85%. Standard policy with ill animals was to withdraw them from the study and place them back on *ad. lib.* feeding until they had recovered before re-entering the study if appropriate using a new baseline as described above.

2.2 The 9 hole box apparatus

The 9 hole box (CeNeS, Cambridge, UK) consisted of a aluminium chamber (25 x 25 cm) with a curved rear wall (Figure 2.1). The chamber was fitted with a wire grid floor and drop tray for cleaning purposes. Access to the chamber was by a hinged Perspex panel on the front wall. The test chamber could be illuminated by a white tungsten filament house light, which remained off unless otherwise noted. Set into the rear wall were 9 equally spaced response holes. All response holes were 2.5 cm square with a 4 cm deep recess behind them

and they were set 2 cm above floor level. A vertical infrared (IR) beam monitored the entrance of each of the 9 response holes. A 4 watt white tungsten filament bulb located at the back of each response hole illuminated it. All response holes could be covered by an aluminium panel. At the front of the chamber a larger access (5 cm long x 4 cm high) led to a recessed magazine. The magazine was also lit by another 4 W filament bulb, and a horizontal oriented infrared beam monitored the entrance to the magazine. All IR beams can be broken by either tongue or nosepoke by a rat and usually by a nosepoke in the case of the mice. Due to the aversive nature of rat odours on mice both mice and rats were never tested in eth same apparatus. In case there were minor variations between equipment set-ups e.g. unusually low stimulus intensities, noisy ventilator fans, etc. each subject used the same equipment throughout training. Steel cannula wire was inserted into the magazine from below and shaped into a shepherd's crook for the mouse experiments only for the delivery of the reinforcer. In the rat set-up the reinforcer was delivered directly into the floor of the magazine. Outside the magazine the cannula wire was connected via silicon tubing to the reward reservoir which contained strawberry flavoured milk (Yazoo, Comelco UK Ltd, Biggin Hill, UK). A peristaltic pump controlled the delivery of milk from the reservoir allowing a ~ 20 μl bolus to be delivered each time. The test chamber and pump system were then mounted in a lightproof sound-attenuating chamber for testing. Behaviour such as grooming, resting and general locomotor actions within the test chambers was observable initially by use of an infrared sensitive camera illuminated by a 0.8 W tungsten filament red light source. Latterly each chamber was equipped with an infrared sensitive camera (Watec 660G 3.8 miniature monochrome camera, Tracksys Ltd, Nottingham, UK) and infrared LED's mounted on the roof of the sound-attenuating box. The apparatus were controlled by an Acorn A5000 computer using software written by Dr. H. M. Marston using the Arachnid real time extension to BBC Basic (Paul Frey Ltd.). Mr. Christopher Spratt and Dr. Marston carried out programme modifications where appropriate. A sample of the programmes used is given in Appendix C. As the test chambers were in sound-attenuating boxes the controlling computer could be kept in the same test room.

Figure 2.1 Plan of 9 hole box.

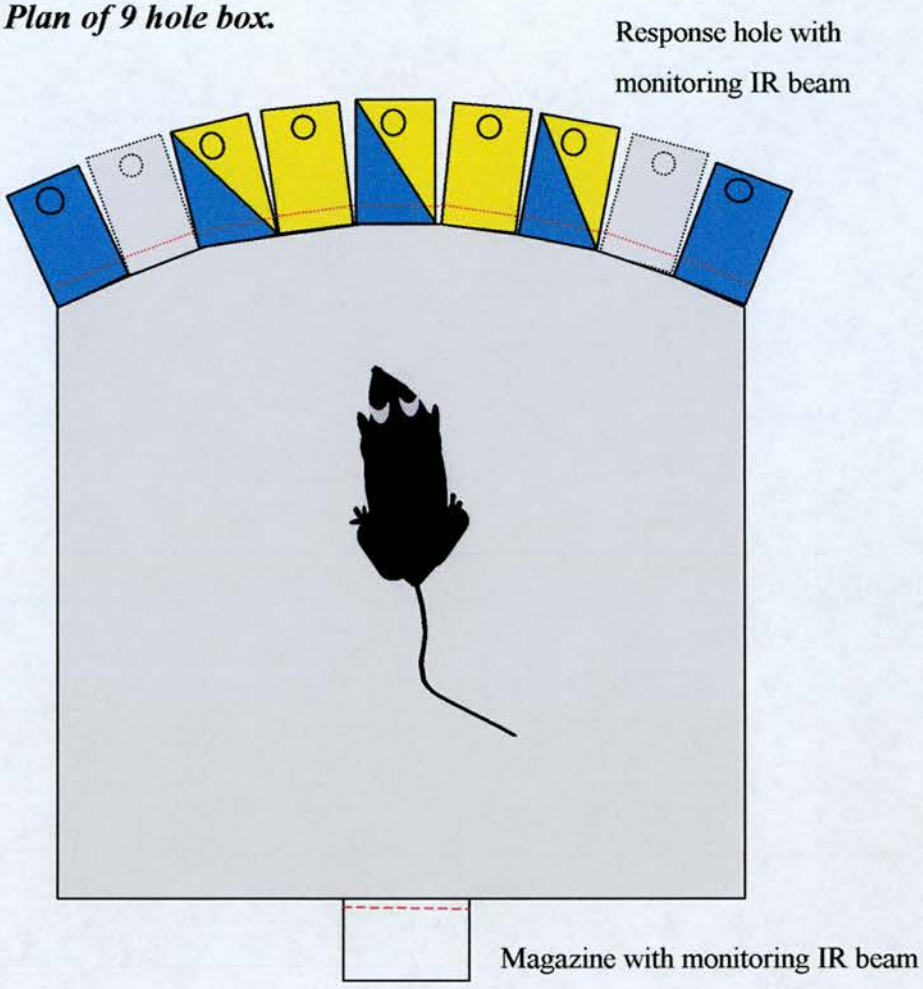


Figure 2.1 shows diagram of 9 hole box. Blue filled response holes indicate pattern of holes used in standard rat training. Yellow filled response holes indicate pattern of holes used in standard mouse training. Grey filled response holes were not used under either standard procedure. Any hole not in use was blocked by a metal panel unless otherwise noted. Red dashed lines indicate infrared beams.

2.3 Wheel running activity

During circadian studies the mice were housed individually in an isolated room from the date of delivery. They were singly housed in clear polycarbonate cages (425 (l) x 266 (w) x 185 (h) mm, floor space 800 cm^2, Tecniplast). Animals were housed under constant dim red light, light intensity below 4 lux measured by light meter (Model RS180-7133, RS Components Ltd., UK) at cage height with a time-controlled white light initially set to lights on 07.00 to 19.00. Mice have not been shown to possess a photopigment sensitive to short wavelengths, which implies mice are relatively insensitive to red light. Therefore the dim red light was sufficient for experimenters to see whilst creating an effective contrast between the

light and 'dark' phases. The circadian experiments were not affected by daylight saving and any study that extended over the switch from GMT to BST or back continued on the original time. The mice were given free access to food and tap water throughout unless otherwise noted. Following agreement with the local Home Office Inspector the mice were given a deep litter arrangement with an average of 30 – 40 mm thickness of wood chip bedding per cage. This allowed subjects to be cleaned out once every four weeks instead of once every week thus minimising handling and disturbance. Water bottles were removed and clean ones replaced every week. Daily visual inspections were carried out according to UK Home Office regulations using a randomised entry time schedule through a lightproof door.

In the first experimental set-up each cage was fitted with a steel running wheel (130 mm diameter, 60 mm wide) attached to the lid. Each wheel was fitted with a metal tag on its circumference. At the 12 o'clock position, the tag protruded through the wire lid and broke the infrared beam mounted on the outside of the lid of the cage. The infrared beam circuit was designed, produced by and used with the kind permission of Dr. M.H.Hastings (MRC Laboratory of Molecular Biology, Cambridge, UK). Assuming the subject ran consistently in one direction the IR beam would be broken once for each full revolution of the wheel, 0.41 m of horizontal travel. Beam breaks were recorded automatically as counts by a PC based Dataquest III interface system (Data Sciences International, St Paul, MN, USA). The Dataquest III v4.10 software collated counts into time-stamped three-minute bins. A second experimental set-up, used only with the $VPAC_2R$ studies, used a different detection system and a larger wheel (240 mm diameter, 80 mm wide) from Tecniplast, Kettering, UK. In this set-up, four miniature magnets were positioned 90° apart on each wheel. These magnets activated a reed relay, which registered counts through a PC based Dataquest ART system (Silver edition, Data Sciences International, St Paul, MN, USA). Each count was equivalent to 189 mm horizontal distance. The software collated counts into time-stamped one-minute bins. The time-stamped data was then downloaded into the MATLAB-based Clocklab software (Actimetrics, Evanston, IL, USA) for pattern analysis.

2.4 Statistical analysis

Parametric analysis of treatments or groups was always carried out following confirmation of normality and equal variance of the raw data (Sigmastat v2.03, SPSS, UK). Where normality or equal variance failed the data was appropriately transformed i.e. log transformation usually but arcsine transformation was used on proportional data. Comparison of data was undertaken with either one- or two-way analysis of variance

(ANOVA) unless otherwise noted, Repeated Measures ANOVA (RM ANOVA) were used where possible. Specific details for any experiment are given in the appropriate results section. *Post hoc* analysis of the data was carried out using Tukey's honestly significant difference (HSD) test unless otherwise noted. Statistical significance was defined by $p < 0.05$.

3 Study 1 Inbred mouse horizontal locomotor activity

3.1 Introduction

In the field of murine molecular biology attention has been drawn back to the surprising variability between commercially available mouse strains. Much of the initial work in murine molecular biology combined widely different mouse strains without much thought for the consequences on the resultant phenotype. Gene knockout technology in particular relied on embryonic stem cells commonly derived from 129 Strain inbred lines whilst utilising the greater breeding potential of strains such as C57Bl/6J for establishment of the resultant lines. Therefore much of the original work was done on mixed genotype backgrounds. Unfortunately, the resultant genetically modified mice were often compared to their respective pure background strains and not their mixed-background littermates. Such confounds advocated extensive investigations into the phenotypes of the inbred strains available. The literature shows a wide diversity of strain responses to a multitude of tasks (a comparatively large list is described by J. Crawley (Crawley, Belknap, Collins, Crabbe, Frankel, Henderson, Hitzemann, Maxson, Miner, Silva, Wehner, Wynshaw-Boris, and Paylor, 1997)). There is considerable evidence that this diversity is further compounded by the experimental conditions under which testing is conducted. Handling has long been reported to be a major factor on animal performance. Living environment and surrounding stimuli can also impact on the phenotype (Williams, Luo et al., 2001) with levels of environmental enrichment being reported to improve recovery from stroke and lesion, increase LTP and hence certain forms of learning. Levels of environmental enrichment are also attributed to improve normal developmental processes. Improved visual acuity (a grating vs. grey cued visual water maze task) has been reported in C57Bl/6 mice raised in an enriched-environment (Prusky, Reidel et al., 2000) compared to mice raised in restricted environment housing.

The prevalence of 129 Strain derived ES cell lines in genetic modification research has led to considerable effort being placed on phenotyping this family of inbred mouse lines. Many reports indicate that the commoner lines such as 129/Sv are less active than many non-129 Strain lines. Following the evidence that environment and handling can have major effects on the expressed phenotype (Miner, 1997;Crabbe, Wahlsten, and Dudek, 1999) it was decided to examine the strains of immediate interest to the laboratory using a simple locomotor assessment.

As a predominantly nocturnal species mice are considered averse to bright lights so the learnt behavioural tasks described here used global exposure to bright light as a punishment. To enhance the effectiveness of this punishment the behavioural tasks were ordinarily under a very low light background by enclosing the test chambers in light-proof boxes. This low level of background illumination might also aid saliency and spatial discrimination of the cue stimuli. Although all behavioural testing was carried out in the light half of the day/night cycle the testing was carried out under low light conditions. Therefore it was deemed necessary to investigate locomotor activity under dim light conditions.

3.2 Materials and Methods

Eight clear polycarbonate cages (480 (long) x 270 (wide) x 200 mm (high)) were fitted with wire grid floors and detachable lids. Drop trays filled with standard wood chip bedding were placed under the wire grid floors for hygiene purposes and to absorb odour markers from test subjects. The cages were then mounted in a two tier rack mounted with infrared light beams positioned perpendicular to the long axis of the cages. Two infrared beam set-ups monitored each cage. The infrared beams were positioned 4 cm from each end of the cage and 2 cm above the grid floor. Beam breaks were registered by a Paul Fray Ltd. detection system attached to an Acorn A5000 computer. Activity was retained, appropriately collated and stored for analysis with software written by Dr H.M.Marston using the Arachnid real time extension to BBC Basic (Paul Frey Ltd.). The software recorded a single activity count each time the two beams were broken in succession. Breaking a single beam repeatedly did not result in any further programmed consequences. The rack and cages were housed in a light controlled, sound-attenuated room whilst the computer was housed separately to avoid distracters like monitor and printer sounds immediately before or during testing.

3.3 Results

Eight C57Bl/6J (C57) and eight 129P2/OlaHsd (129P2) inbred mice were tested. All subjects remained on *ad. lib.* food and water throughout and testing was done in the middle of the light phase of their normal LD cycle. As with other studies presented here the subjects were tested under dim light conditions (room illuminated with a single fluorescent strip light under a red filter). They received three consecutive daily sessions with each session lasting for 100 minutes, divided into ten, 10 minute time-bins for data collection purposes. Activity levels were analysed using Three way ANOVA of strain x session x time bin to determine any differences. There was a significant main effect of strain ($F_{(1,420)} = 133.5$, $p < 0.001$) and of

time bin ($F_{(9,420)} = 8.0$, $p < 0.001$) but no significant main effect of day ($F_{(2,420)} = 2.2$, $p = 0.11$, n.s.) using logged data. Therefore the data over the three days was collapsed by session (Figure 3.1). *Post hoc* analysis confirmed that the C57 strain was significantly more active than the 129P2 strain and that overall, subjects were significantly more active in the first time bin, however this is predominantly due to the initial activity burst of the 129P2 mice.

Figure 3.1 Effect of strain on horizontal locomotor activity

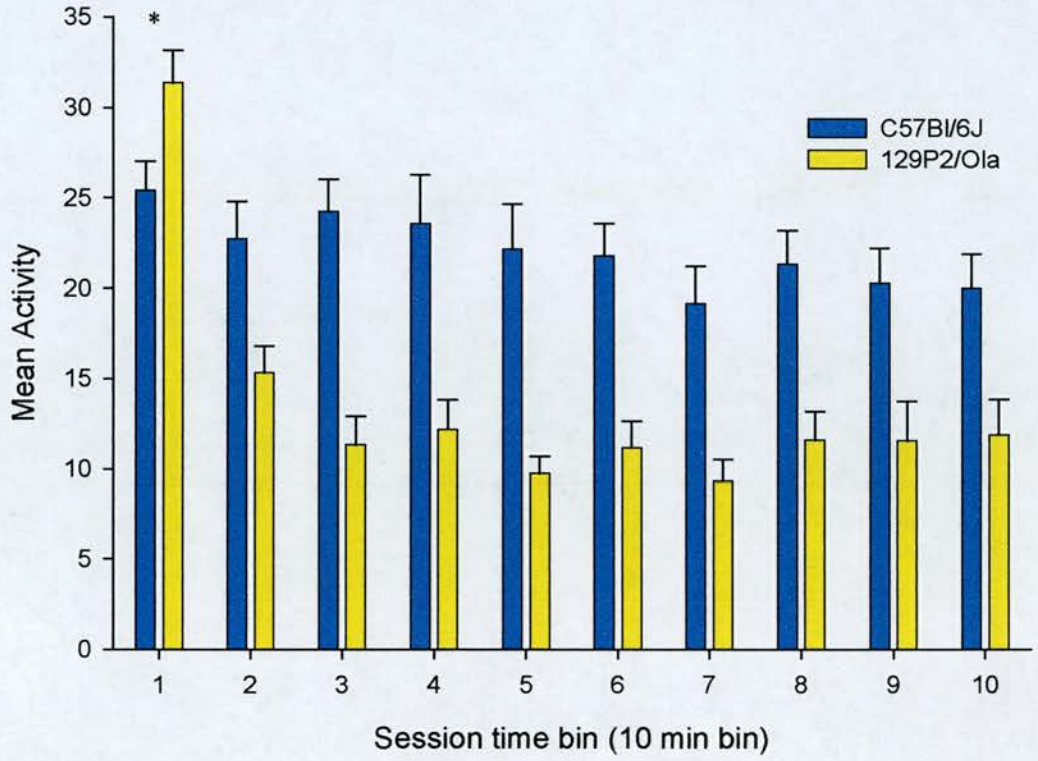


Figure 3.1 Data is plotted from the mean ± SEM of three test sessions. * indicates significant difference ($p < 0.05$) from all other time bins.

3.4 Discussion

This data supports the literature evidence (Miner, 1997; Royle et al., 1999), which noted the 129 substrains were less active than many other inbred mouse strains in locomotor tasks. This phenotypic characteristic may impact upon the acquisition of complex behaviours and so must be taken into consideration with subsequent experiments described in this thesis. The pattern of activity shows the 129P2 mice were very active in the first time bin but this fell rapidly to a stable level throughout the rest of the session. This is suggestive of either increased anxiety following handling or an arousal-induced response to being introduced to a

novel environment. The rapid fall-off suggests a rapid habituation to the novel environment. In contrast the C57 mice showed only a slight reduction in activity over time, which may result from either a comparatively elevated endogenous locomotor activity or a failure to habituate to the handling and the arousal resulting from transference to the test chambers. The transfer of the subjects into the dimly lit test room may have also contributed to their arousal state. However, since all subsequent operant tasks were to be carried out in dimly lit conditions this is an acceptable, indeed necessary behavioural response. The absence of decreasing activity over the three days suggests that there was no habituation to the behavioural protocol itself and that the handling and transference arousal had similar effects if any throughout the experiment. Homanics et al reported no difference in the total activity of C57Bl/6J versus 129/SvJ mice during an open field test without prior handling experience. However, a second group of mice did show a difference between the two strains, with the C57Bl/6J mice being more active than the 129/SvJ group. In this second cohort all the mice were reported to have had extensive handling in the weeks before the open field test. Of particular note is that the C57 group showed comparable activity to the initial handling-naïve animals whereas the handled 129/SvJ mice showed nearly half the activity of their naïve counterparts. This strongly suggests that the persistently elevated activity in the C57 mice reported in this thesis is not an arousal-induced effect. The data also suggest that although the 129P2 mice appear to be more susceptible to handling-induced arousal this does appear to be a short-term effect and would abate within the intended duration of the operant tasks described here.

4 Study 2 Inbred mouse running wheel

4.1 Introduction

With the expansion of molecular biological techniques one of the areas of interest has been dissection of the molecular clock. As has been already noted mice still provide one of the most stable platforms for this genetic manipulation work. It is now widely accepted that there are radical differences within the inbred mouse strains for many behaviours. To assume there are no differences in circadian biology would be naïve. Following the demonstration of a significant difference between the spontaneous activity of the C57 and 129P2 groups after handling arousal and exposure to a novel environment (see Chapter 3) it was necessary to investigate these strains in a true circadian behavioural study. The circadian studies presented utilise the spontaneous behaviour of many small rodents to use freely moving wheels if provided. Correlating the intensity of wheel movement to the timing of such activity is an accepted method of characterising the overt circadian behaviour of a rodent. Like many other rodents mice are predominantly nocturnal mammals. As such they display a marked polarisation of activity so that under standard laboratory conditions they become very active within 1 hour of lights out. This phenomenon is called activity onset and is taken as the onset of perceived night.

4.2 Materials and Methods

In this study groups of eight C57, 129P2 and CBA mice were singly housed in a light controlled room, light phase 07.00 to 19.00 BST. The schedule of light manipulations is given in Table 4.1, the various stages of the study being defined by the timing of exposure to the white room light. The initial acclimatisation phase was required as the apparatus was very sensitive to mild deformations in the wheel and many small adjustments were necessary to optimise signal to noise levels. These invariably necessitated disturbing the mice and hence that phase is clearly separated from the rest of the study. The shift from the standard 07.00-19.00 light phase to 23.00-11.00 light phase required an 8 hour shift in light cycle. The day labelled dark phase advance required the lights to switch on at 07.00 as normal for Phase 1 and then be switched off at 11.00, 8 hours earlier than normal. The lights then stayed off for 12 hours and then came on again at 23.00. This established the secondary LD cycle Phase 2. The shift back to the standard LD cycle was conducted by extending the light cycle, thus delaying the dark phase by 8 hours. The parameters measured were the mean activity in both

light and dark conditions under a normal 12 hour LD cycle and the level of activity expressed in constant dim red light (DR). Light and dark activity was calculated from the activity profile using a 24 hour cycle (data generated by the Activity Profile function of the Clocklab software). An individual's activity profile was generated from the mean activity over a period of days under stable conditions, the final days of the initial LD cycle in this case. Mean total activity was then calculated from the 12 hours in light cycle and 12 hours in the dark cycle. Activity in constant darkness also used the activity profile function but due to the nature of free-running activity it was decided to look at total activity over the entire 24 hour period. Patterns of activity throughout the experiment were plotted by double plot actograms as shown in Figure 4.1. Each actogram plots the timing and intensity of a single subject's activity over a 48 hour period horizontally. Except the first 24 hour period each day's data is also replicated vertically in the two activity columns seen. This vertical plotting allows the experimenter to see changes in the onset of activity. The 48 hour horizontal plot allows room for observing long-term drift in the time of onset. The ability to adapt to a novel LD cycle was examined by an 8 hour advance and an 8 hour delay in the onset of darkness. The number of days taken to re-entrain was estimated from analysis of the actograms by comparing time of activity onset with the new time of darkness onset. Time of activity onset and offset were determined by Clocklab software by comparing total activity ratios for the six hours preceding and following every 3 minute bin. Onset time was then suggested by the software based upon most likely period of inactivity followed by sustained activity. Offset time used a reciprocal process. If the time of activity onset equalled the current time of darkness onset the subjects were deemed to have successfully re-entrained. Reproducibility was defined by a subject demonstrating a stable activity onset time equivalent to darkness onset over consecutive days, this ruled out any novelty responses. Re-entrainment time was taken from the first day of stable entrainment. Re-entrainment time was measured in whole days using a minimum value of one day if the subject managed to re-entrain within the initial 24 hours following the shift. Free-running period tau (τ) was calculated using a line fitting function (Clocklab software) to the time of activity onset from a 10 day period in constant darkness. The period of DR used was the same for assessment of activity expression and tau determination.

Figure 4.1 Structure of an actogram display

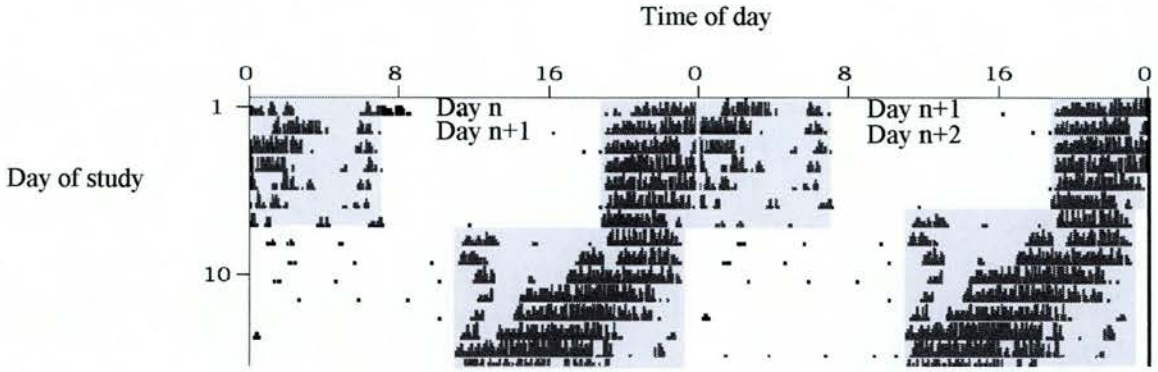


Figure 4.1 shows the design of a sample double plot actograms for an individual mouse. The sample shown is taken from part way through the full test schedule and thus does not adhere to the schedule listed in Table 4.1 below. Activity is autoscaled to the maximum activity of the subject over the entire study. The y–axis plots day of study and the x-axis plots time of day; 0 equal to midnight 00:00. Each row represents 48 hours of activity with the second 24 hours data replicated at the start in the subsequent row throughout as shown. Periods of darkness (dim red light) are indicated by shaded areas.

Table 4.1: Inbred mouse light exposure schedule.

Duration (days)	White light on	Comments
7	07.00 19.00	Equipment tuning and acclimatisation
10	07.00-19.00	Phase 1, entrainment to standard LD cycle
1	07.00-19.00, 23.00-11.00	8 hour Dark phase advance
22	23.00-11.00	Phase 2, entrainment to secondary cycle
1	00.00-19.00	8 hour Dark phase delay
8	07.00-19.00	Phase 3, re-entrainment to standard cycle
23	Constant darkness	Phase 4
4	07.00-19.00	Phase 5, re-entrainment to standard cycle

Table 4.1 shows schedule of exposure to white room light in inbred mouse strain study.

4.3 Results

Mean locomotor activity was calculated as the mean of a ten day period in either the 12 hr light or dark phase. RM ANOVA showed a significant effect of phase ($F_{(1,21)} = 37.82$, $p < 0.001$) but no effect of strain ($F_{(2,21)} = 2.47$, $p = 0.109$ n.s.) although the C57 group were most active in both phases. *Post hoc* analysis showed a significant increase in activity in the dark phase in comparison with the light phase. To compare the effect of an absence of light on daily activity, total daily activity was calculated over 10 days in constant darkness. This was compared with total daily activity over 10 days under the standard LD cycle. Analysis showed the level of activity was dependent on the regular presence of light ($F_{(1,21)} = 43.07$, $p < 0.001$ but was independent of strain ($F_{(2,21)} = 2.47$, $p = 0.109$ n.s.) using RM ANOVA. *Post hoc* analysis showed that the mean total activity increased under constant dark conditions.

Figure 4.2 Sample C57Bl/6J actogram

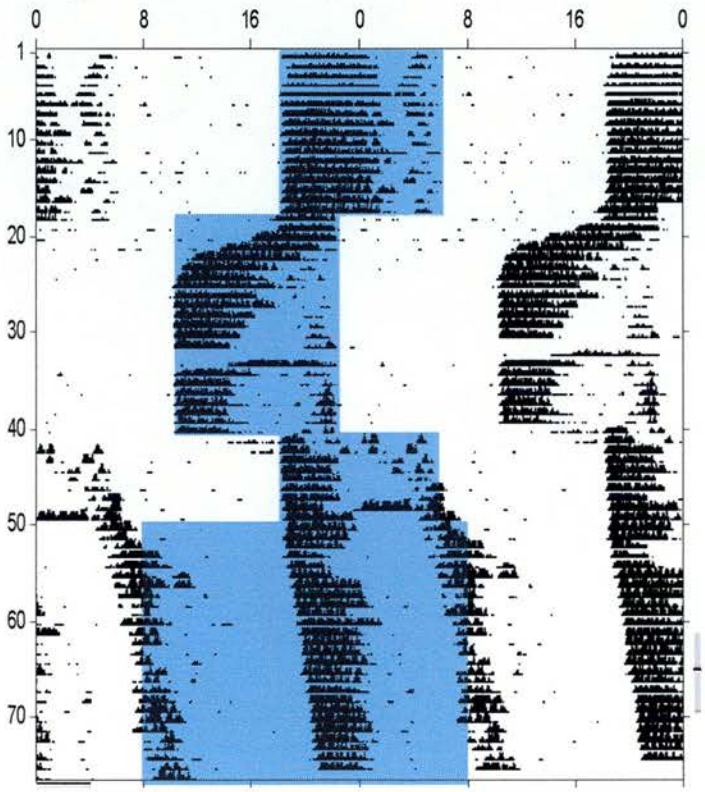


Figure 4.2 A sample C57 actogram is plotted, activity is autoscaled to the maximum activity of the subject over the 76 day study. Periods of dim red light are indicated by the shaded area. This is only shown for one cycle.

Figure 4.3 Sample 129P2/Ola actogram

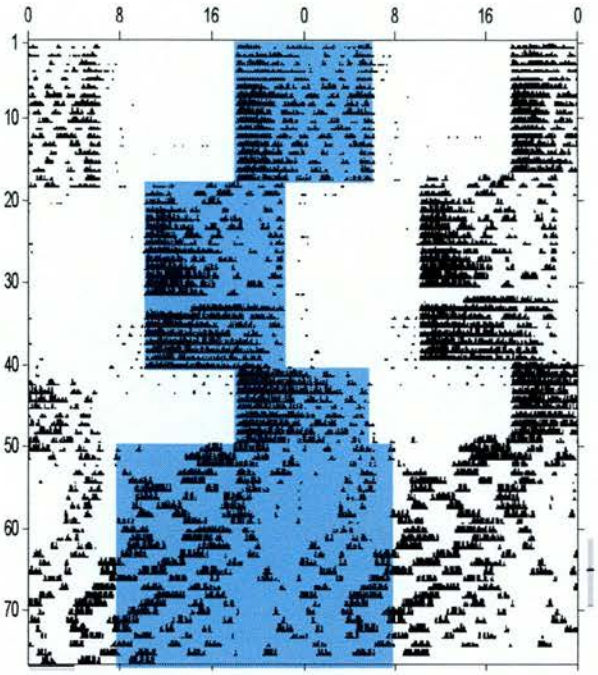
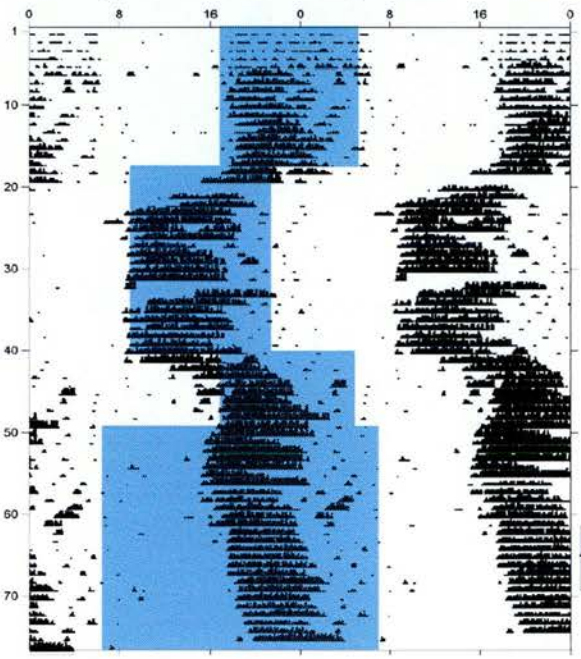


Figure 4.4 Sample CBA/Ca actograms



Figures 4.2 to 4.4 show the actograms for single subjects only. The actograms for all 24 subjects (8 per strain) are shown in Appendix A.

Figure 4.5 Inbred Diurnal vs. Nocturnal activity

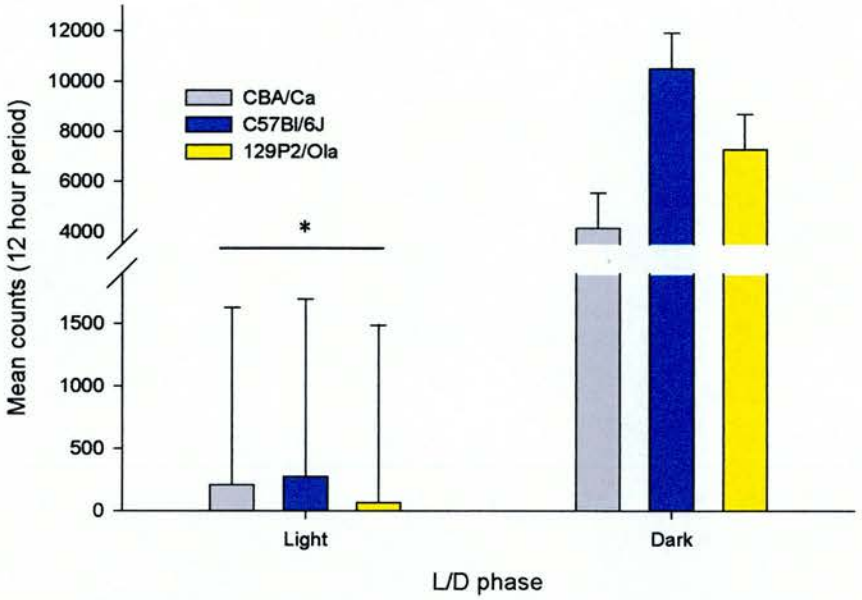


Figure 4.5 shows the total running wheel activity over a 12 hour period in light (Light phase) or dark (Dark phase) by strain. Data is plotted as mean ± SEM of ten days from 8 subjects per strain. * indicates significant difference between phases independent of strain.

Figure 4.6 Inbred LD vs. DR activity

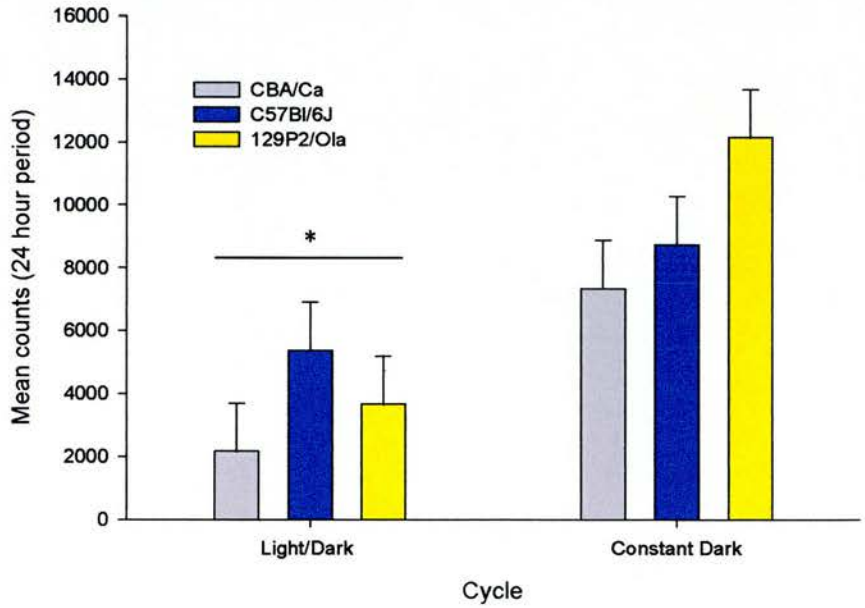


Figure 4.6 shows total activity over a 24 hour period by strain. Light/Dark data is mean of total light phase and total dark phase activity. Constant dark data is derived from total activity over 24 hour period. Data is plotted as mean ± SEM of ten days from 8 subjects per strain. * indicates significant difference between cycles independent of strain.

The imposition of a novel LD cycle caused all strains to re-entrain over time. Advancing the light cycle 8 hours resulted in a gradual shift to the new time of lights off in all strains. One way ANOVA of the days taken to re-entrain showed this was strain-dependent ($F_{(2,21)}$ = 21.68, $p < 0.001$). *Post hoc* testing showed that the 129P2 group shifted significantly faster than either of the other strains. The reciprocal shift, an 8 hour delay in darkness onset, also resulted in a strain dependent re-entrainment ($F_{(2,21)} = 7.11$, $p = 0.004$). *Post hoc* analysis showed that whilst the 129P2 group again exhibited rapid entrainment the C57 group also re-entrained rapidly. However, the CBA group re-entrained significantly slower than either of the other two strains. Analysis of the endogenous free-running rhythm of these strains also showed a significant strain effect, ($F_{(2,19)} = 4.23$, $p = 0.03$) using the 10 day period previously used for assessing DR activity. *Post hoc* analysis showed that the C57 strain had a significantly longer tau than the 129P2 group. However, the power of this one way ANOVA was low implying this is a weak difference. A larger sample size may have given clearer resolution.

Figure 4.7 Inbred days to re-entrainment

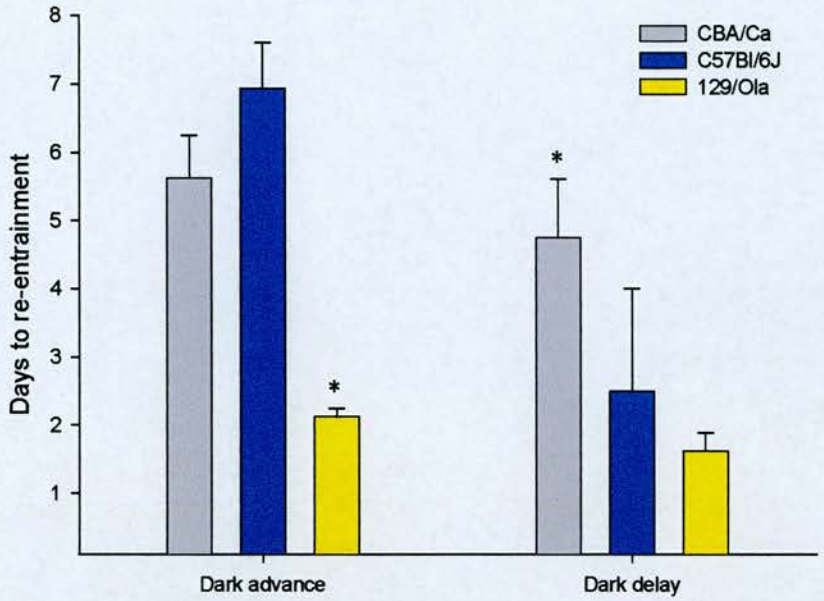


Figure 4.7 shows the days taken to stably re-entrain to novel shifts in the LD cycle measured by an 8 hour advance or delay in the onset of darkness. Data is plotted as mean ± SEM of 8 subjects per strain. * indicates significant difference from other strains at that challenge.

Figure 4.8 Inbred Free-running period

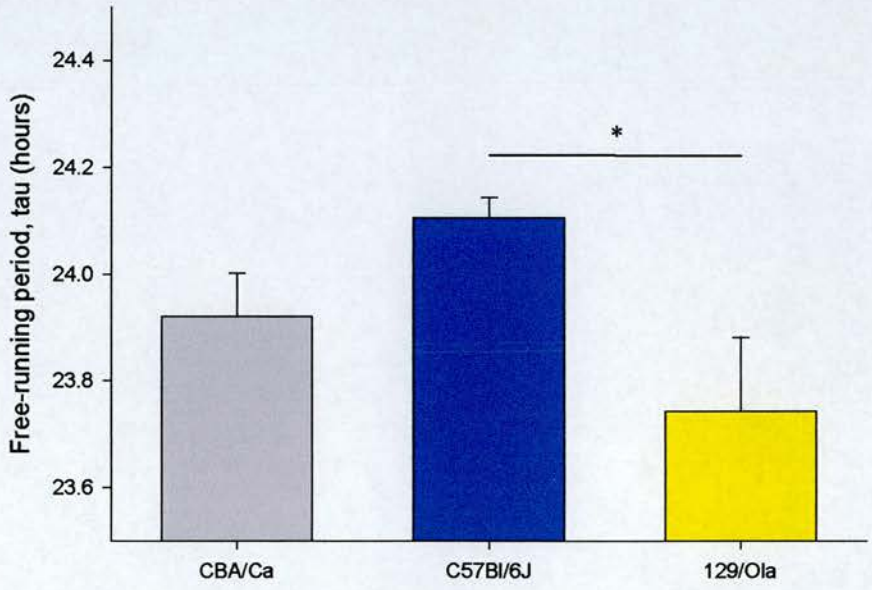


Figure 4.8 shows tau, the free-running period of the three strains. Tau is derived from the periodicity of onset of activity over ten days in constant darkness. Data is plotted as mean ± SEM of 8 subjects per strain. * indicates significant difference between the marked strains.

4.4 Summary

There are limitations with the approach used here for comparison between the activity under the presence and absence of light. The mean activity over the 24 hour LD cycle is depressed by the 12 hour period under light suppression. However just using the 12 hour darkness period from the LD phase would be unacceptable as that could ignore any effects of non locomotor behaviours such as feeding and sleep which necessarily occur during the entire 24 hour period used for constant darkness analysis. Selecting a 12 hour period in constant darkness would also prove unsuitable as any attempt to select for the active period equivalent to the dark phase in LD is necessarily skewing the data and the sampling is no longer random. The approach used here at least demonstrates the suppressive effects of light. It is of note that whilst no strain effects were seen in this experiment the C57 strain was most active under both phases of the LD cycle but under DR the 129P2 strain was the most active strain. The other indices used, tau and rate of re-entrainment yielded strain differences that indicate the 129P2 group adjust much faster to changes in the light environment than the other strains.

4.5 Discussion

The data confirm that there are strong differences between the strains tested. Some of these differences could be anticipated based on the literature reports of relatively inactive 129 strains but nothing has been mentioned previously about other responses to light stimuli. Following the literature on mouse activity levels and the horizontal locomotor activity data shown here it was anticipated that the 129P2 strain would be less active than the C57 strain. This was seen within the LD cycle data but surprisingly the reverse was seen in DR. Under constant darkness the 129P2 were much more active than the C57 group (Mean activity counts/day = 12143 for 129P2 vs. 8737 for C57). One possible explanation is that the 129P2 group is strongly suppressed by exposure to light. The C57 strain express an equivalent of 3% of their total nocturnal activity during the day, the CBA mice express an even higher proportion at 19 % of their nocturnal activity. The higher proportion of activity during day seen in the CBA strain is due in part to a greater variability around the time of darkness onset. This suggests that the CBA strain is less affected by the suppressive effects of light. The 129P2 mice show an equivalent of less than 1 % of their nocturnal activity during daylight hours. As has already been noted this suppressive effect strongly depresses the mean daily activity under LD in comparison to DR. This led to nearly a fourfold increase in total activity under DR in the 129P2 group in comparison to a less than twofold increase in

the C57 mice. However, the horizontal locomotor activity presented earlier showed that merely an absence of strong white light was not sufficient to generate a massive increase in activity because the horizontal activity was measured under dim red light as well. Inspection of the actograms (see Appendix A) suggests that the increase in overall activity under DR conditions in the 129P2 group is due to an increase in the active period (alpha period) or a decrease in the inactive period (rho period) in these mice. The C57 group tend to retain a stable rhythm in DR with the alpha period and rho periods roughly equivalent to that seen in LD, in contrast the 129P2 group show a sharp decline in the length of the inactive period and a broadening of the alpha period. Since the data in Chapter 3 is only taken under dim red light and not also under normal white light it is impossible to categorically state that the brief exposure to dim red light did not result in an increase in activity over and above that initiated by exposure to a novel environment.

5 Studies 3 and 4 Single choice progressive ratio task

5.1 Introduction

The operant paradigms described here utilise appetitive conditioning rather than aversive conditioning such as foot shock in order to motivate subjects to perform the tasks. Previously a food restriction protocol has been used in rats to establish an adequate motivational state (Carli, Robbins, Evenden, and Everitt, 1983). However, water restriction has been described to work well in mice in appetitive tasks (Humby, Laird et al., 1999;Chapman, White et al., 1999). It was decided to investigate the efficacy of the two different strategies using a simple operant protocol using the 9 hole box described in Section 2.2.

5.2 Study 3 Restriction methodology comparison

The initial two stages described were designed as standard habituation steps in the operant protocols used here.

5.2.1 Habituation to the reinforcer

This phase was to introduce a reinforcer (strawberry flavoured milk) to the subjects in their home cage. This was done by applying milk to the cage lid bars or inside walls of the cage near the mice. This was continued in short bouts (approximately 5 minutes each bout) at 30-minute intervals until the mice spontaneously sampled the proffered milk. A subsequent adaptation to this approach was to replace their water with milk or milk/water mix overnight preferably whilst still on *ad. lib.* feeding. Once all animals were sampling the milk they were moved onto the next stage of training.

5.2.2 Habituation to the test equipment.

This phase was designed to both introduce the subjects to the test chamber and to form a location – reward association by introducing the concept that milk reward, introduced above, can be obtained in the magazine of the 9 hole box. A programme was written that delivers 20 μl milk reward every 15 seconds into the magazine. The box was kept dark with the exception that the magazine light was activated every time milk was delivered to the magazine. The magazine light was extinguished if the magazine IR beam was broken. The

programme recorded the number of times the magazine light was extinguished. The standard session was 10 minutes so with a 15 second inter trial interval (ITI) the maximum count could be 40. Criterion for moving onto the next stage was having a count of 30 or greater and no milk left in the magazine.

5.2.3 Single choice progressive ratio task

A single choice progressive ratio study was designed to investigate the subjects' capacity to repeatedly respond in order to get reward. The 9 hole boxes were configured to leave a single central response hole uncovered. The Arachnid software was heavily modified from the earlier 5CSRT programmes to create a progressive ratio response task. The convention is used that an activated hole is also a lit hole unless otherwise noted.

A schematic of the progressive ratio task is indicated in Flowchart 5.1 and the details are listed below. The progressive ratio task began with the central response hole being activated. The hole remained active until the subject had made a predetermined number of beam breaks (called the fixed ratio, FR) at that location. The time taken to complete the FR, measured as the time elapsed between initial beam break and the final beam break was recorded. Target completion latency was determined according to the calculation given below.

Equation 5.1

$$\text{Target Latency} = (1.5 + [FR/2])$$

This gave a target latency (in seconds) where FR was the current fixed ratio, e.g. at a current FR of 4 the target latency would be 3.5 sec. The actual ratio completion latency was then compared with the target latency. To increase the task difficulty and give some indication of motivational state the FR was incremented by one depending on performance over the recent trials. If the completion latencies for the previous **n** consecutive trials were all equal to or below the target latency for that FR then the FR for subsequent trials was incremented. The number of consecutive trials required (n) was an externally enterable variable initially set to 20 trials but later reduced to 10 trials. Failure to complete an ratio within the current target latency on any particular trial resulted in a reset to the number of trials required for increment of the FR. Any increment in FR would result in a recalculation of the subsequent target latency. Once a subject had completed the current FR, the central response hole was inactivated. Reward was then delivered to the now activated magazine. Breaking the

magazine beam inactivated the magazine and started the inter trial interval (ITI). After the ITI was completed the central response hole was reactivated and the next trial begun. Initially a single beam break was required to complete the FR but since there was no possible completion latency for an FR 1 trial the subject would automatically reach FR 2 after **n** trials.

Figure 5.1 FR incrementing protocol

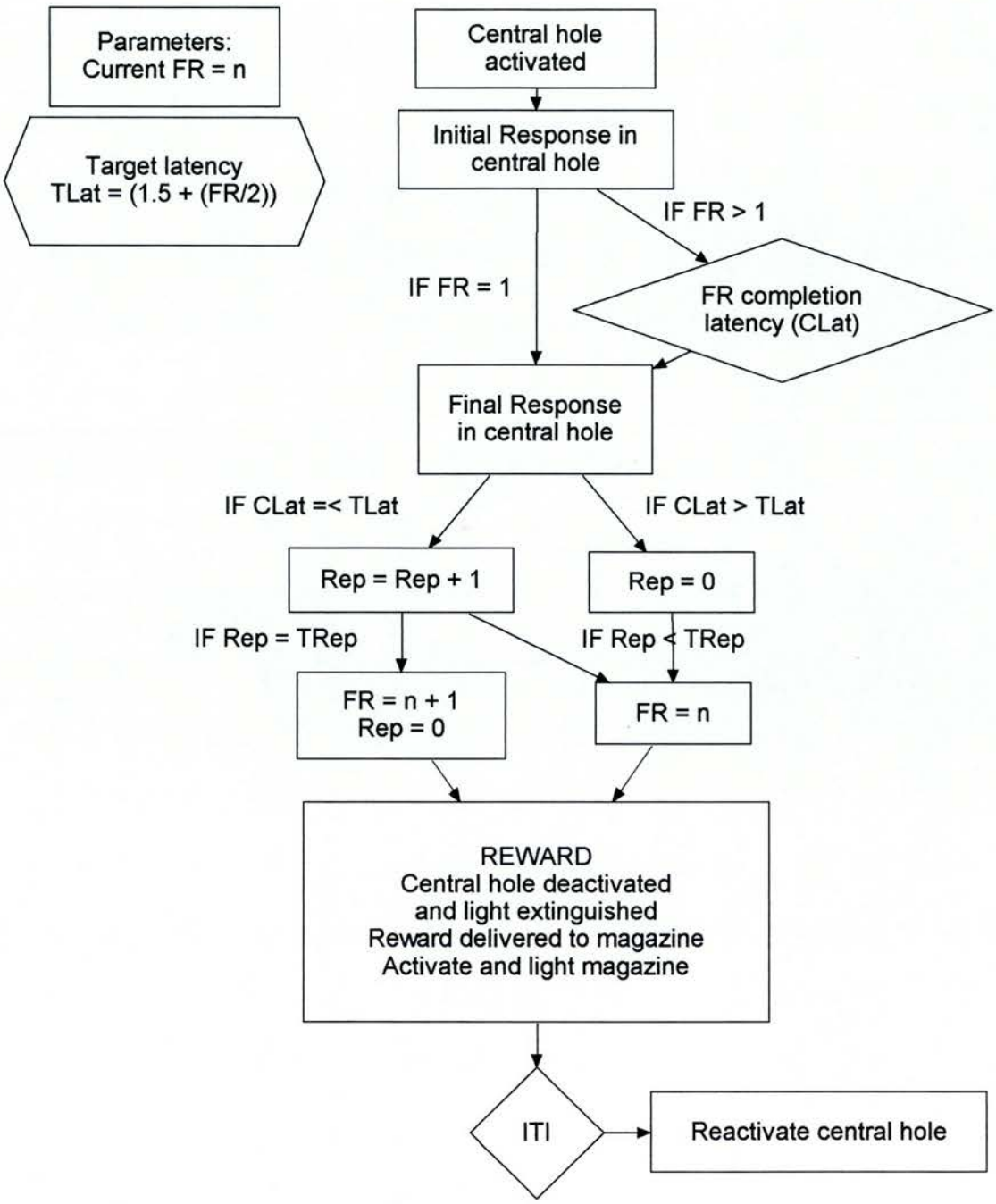


Figure 5.1 shows the progressive ratio routine. FR is the current Fixed Ratio; Clat is the current FR completion latency. Rep. is the current number of consecutive trials (repetitions) completed under the current Target completion Latency (Tlat). TRep is the Target Repetitions, the number of consecutive trials to be completed within the target latency in order to increment FR. ITI is the inter trial interval. See description above for more details.

Sixteen male C57 mice were used, all subjects were initially trained using a low FR dependent version of the task under the food restriction protocol (Section 2.1.3). **N** was set to 20 so that 20 consecutive trials were required before the FR was incremented. This allowed all subjects twenty trials at FR1 per session before having to move to the more difficult FR2. After this initial training the subjects were evenly distributed between food and water restriction protocols, with prior levels of performance also being balanced across both protocols. Water restriction was as described (Humby, Laird, Davies, and Wilkinson, 1999) with modifications. Water restriction involved free access to food but limited daily access to water to 90 minutes post testing. All subjects were weighed daily and carefully monitored to prevent deteriorating health. Water and food access was carried out 15.00 to 17.00 weekdays and 09.00 to 11.00 at weekend. After 8 days acclimatisation to the restriction protocols the animals began testing.

All subjects were placed on the standard habituation to test equipment protocols (Section 5.2.2.) for two days prior to moving to the progressive ratio task listed above due to the break in training. The progressive ratio task ran for 20 minutes or 60 trials completed with 10 consecutive trials required for FR progression. Performance in the progressive ratio task was assessed by analysis of the number of trials taken to initially reach each of the fixed ratios, the total number of responses made (i.e. the sum of all the ratios responded to over a testing session – equal to the minimum number of nosepokes at the response hole made) and the number of subjects to reach each FR over the initial three weeks.

5.2.4 Results

Performance was assessed by the number of trials taken to initially reach each FR (Figure 5.2). Analysis excluded any fixed ratio that a subject failed to reach, in this case all subjects reached up to FR 3. Only 50 % of water-restricted subjects reached FR 4 within the three weeks. Because of this it was felt that inclusion of only the FR's that all subjects reached gave the least biased comparison. RM ANOVA showed a strong effect of FR level ($F_{(1,14)} = 26.7$, $p < 0.001$) and of restriction protocol ($F_{(1,14)} = 6.31$, $p = 0.025$) and a weak interaction between the two effects ($F_{(1,14)} = 7.4$, $p = 0.016$) at these lower ratios using logged data. *Post hoc* analysis showed the water-restricted group required more trials to reach the higher fixed ratios but this became significant only at FR 3.

Figure 5.2 Trials to FR

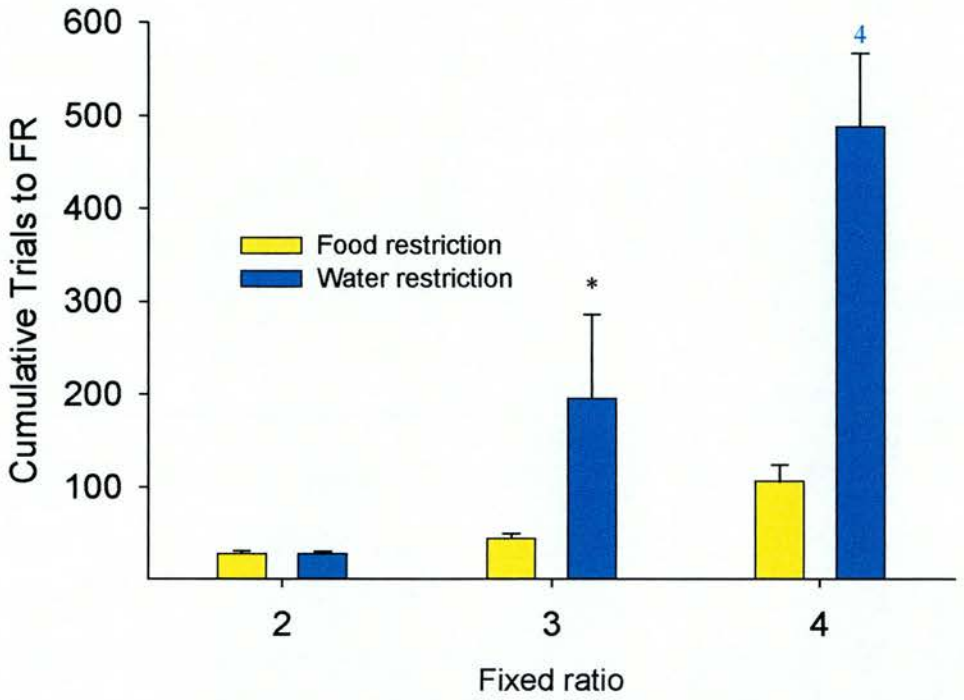


Figure 5.2 shows cumulative trials to reach each fixed ratio (mean ± SEM, n = 8 unless otherwise noted on the graph). * indicates a significant difference between restriction protocols, $p < 0.05$ was considered significant.

Calculation of the total number of central hole responses made during a session also showed significant differences between the two groups (Figure 5.3). Effects of group ($F_{(1,196)} = 49.62$, $p < 0.001$), day ($F_{(14,196)} = 3.37$, $p < 0.001$) and an interaction between the two main effects ($F_{(15,196)} = 3.22$, $p < 0.001$) were all seen using RM ANOVA on the logged data. As can be seen the response levels in the water-restricted group never reached the food-restricted group's response levels. Plotting of the total response data showed several rhythmic peaks in the water-restricted group's performance. Further inspection showed that each of these peaks occurred on a Monday.

Figure 5.3 Total responses

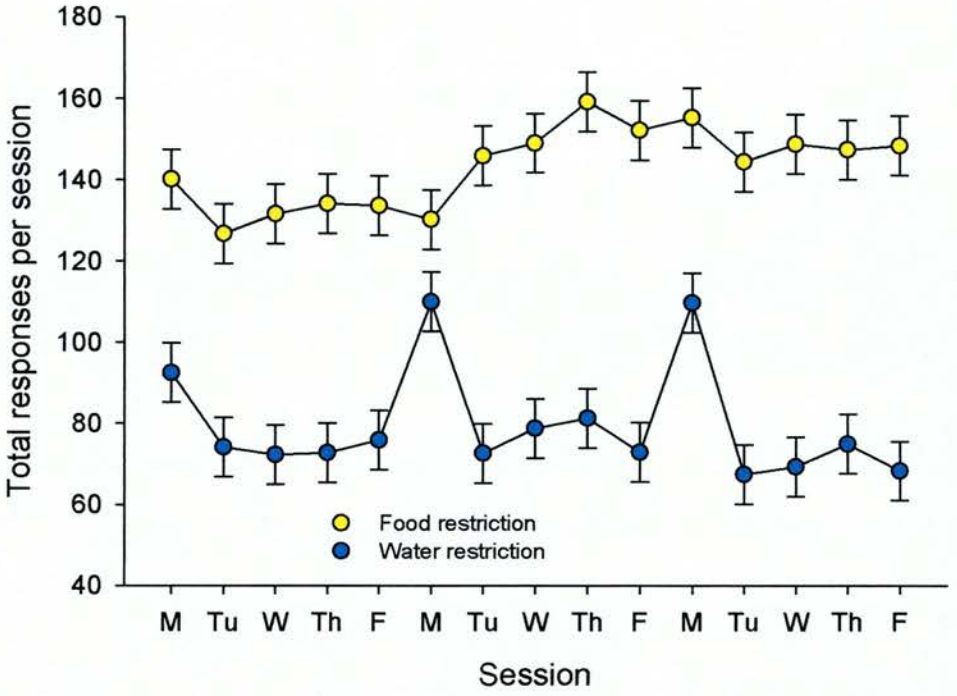


Figure 5.3 shows the total responses over session (mean ± SEM, n=8 per group) made by the food-restricted and water-restricted groups.

Collapsing the data from these initial three weeks of the study by day showed main effects of day and treatment group, effect of day ($F_{(4,226)} = 3.122$, $p = 0.016$), effect of group ($F_{(1,226)} = 320.65$, $p < 0.001$) and a significant interaction between the two, interaction ($F_{(4,226)} = 2.851$, $p = 0.025$) using two way ANOVA. *Post hoc* analysis showed that mean responses were significantly lower in the water-restricted than in the food–restricted group on all days (Figure 5.4). Furthermore, within the water-restricted group alone response levels were significantly higher on Monday than on any subsequent day. The number of subjects to reach each FR was also examined, using Fishers Exact test as the group sizes were small. Comparisons of the number of subjects to reach each FR demonstrated there was a significant difference between the two restriction groups for reaching FR 6 ($p = 0.026$) and FR 5 ($p = 0.041$) but not FR 4 ($p = 0.077$) or below.

Figure 5.4 Effect of day

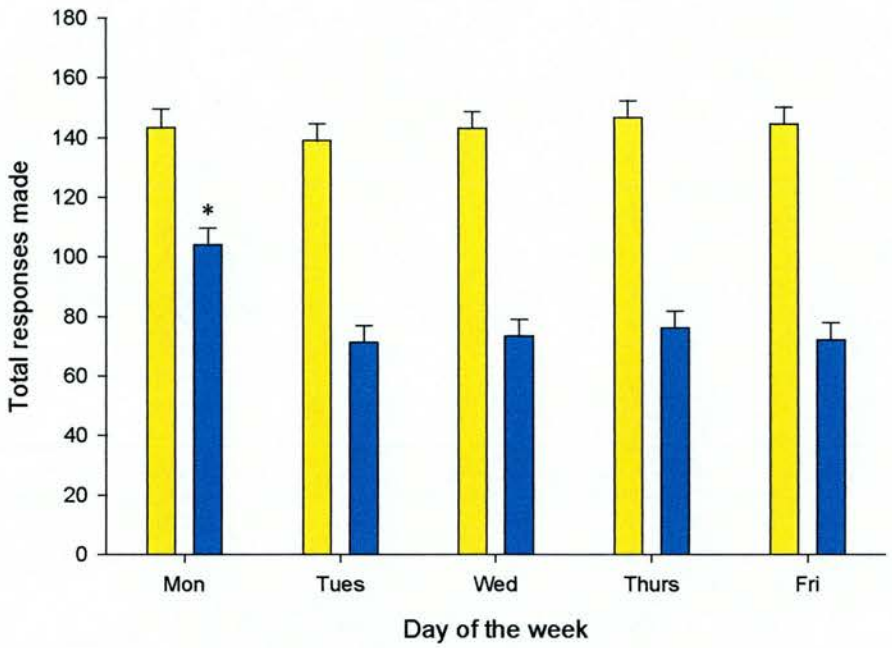


Figure 5.4 shows the day dependent effect of method of restriction. Plotted are the total number of responses made (mean ± SEM) over the initial three weeks under the dietary restriction comparison. As with the previous figures in this chapter the water-restricted group is plotted in blue. * indicates a significant difference from all other days within the water-restricted group alone, $p < 0.05$ was considered significant.

The day-dependent effect is a motivational artefact presumably derived from the subjects' weights under the two restriction protocols. Plotting of proportionate weight demonstrated that the food-restricted subjects were successfully maintained at approximately 87% of their assumed free-feeding weight. The water-restricted mice however did not show any decrease over free-feeding weight, in contrast their weights increased over the course of training. However, there was a significant decrease in mean weight on each Monday of testing in the water-restricted group alone (Figure 5.5).

Figure 5.5 Proportionate weight.

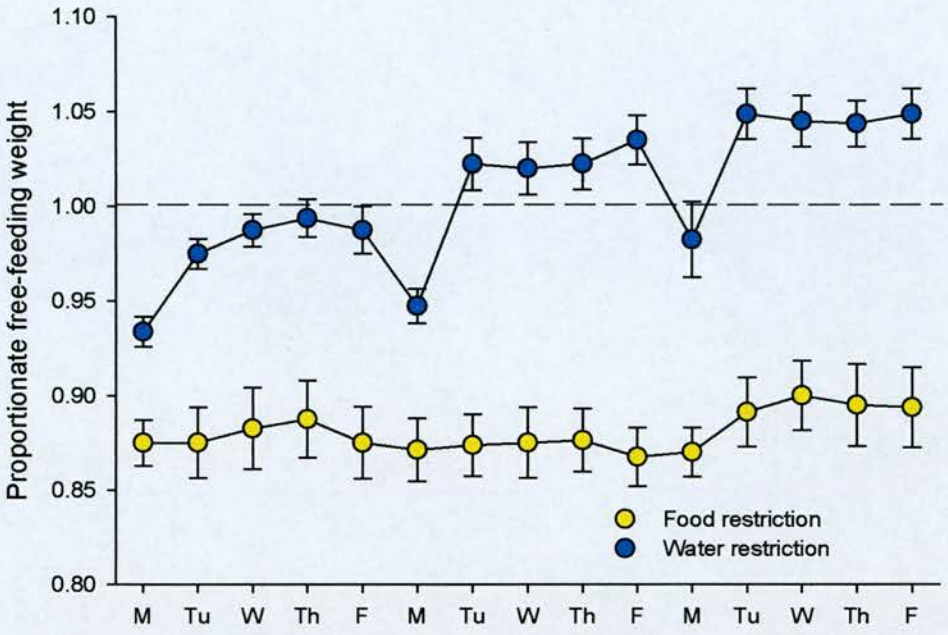


Figure 5.5 shows proportionate weight (calculated as proportion of predicted free-feeding weight, indicated by dotted line), mean ± SEM of 8 per group.

5.2.5 Discussion

The comparison study showed that in our hands food restriction was significantly better than water restriction for our purposes. The food-restricted mice attained the higher ratios more swiftly with a concurrent increase in the number of responses made. Analysis of the weights of the food-restricted and water-restricted mice gave some indication as to why there was a significant treatment effect. The food-restricted animals were always near 90% free feeding weight, as food was titred to maintain this. In contrast, the water-restricted animals remained at or above baseline free-feeding weight, in this case free-feeding weight being defined from the period with free access to both food and water. The significant drop in the water-restricted mice's weight each Monday provides an explanation for the concurrent improvement in performance noted. This improvement is likely to be a result of the weekend husbandry regime when water was made available 6 hours earlier than during the week. This additional 6 hour period seems to have been critical in their performance, however since food and water were made available immediately after testing it was impossible to duplicate this additional 6 hour period every weekday. Such sensitivity to the duration between access

and subsequent testing was not favourable so water restriction was deemed to be insufficiently robust to use in the appetitive tasks presented. There was no equivalent drop in weight in the food-restricted group because the standard food restriction protocol included an increase in food to accommodate shifts in feeding time. The lack of such an adjustment in the water-restriction protocol was an oversight, which fortunately gave additional information regarding the effects of weight on response levels. The total responses made was considered to be an index of motivational state of the subject, disinterested or poorly motivated subjects may complete all 60 trials but would be expected to remain at a low FR, measuring responses made gave an indication of the effort expended in getting the reward.

5.3 Study 4 Breakpoint study

5.3.1 Introduction

Following the demonstration that food restriction provided the most stable performance it was decided to examine how high a fixed ratio the subjects could attain under food restriction only. This was deemed of interest as the initial protocol limited the mice to reaching an FR 6. Furthermore, moving all subjects to food restriction would demonstrate whether the reduced performance seen in water-restricted subjects was reversible.

5.3.2 Methods

All mice from Section 5.2 were returned to *ad. lib.* water and restricted feeding. To reduce the ceiling effect seen by forcing the mice to complete 10 consecutive trials the number of repeats required was reduced to 5 repeats. With a session limit of 60 trials this allowed subjects to reach a maximum of FR 12. Due to the varying final levels of performance in the food vs. water experiment it was necessary to analyse the animals according to their separate groups even though now they were all treated equally. Data was obtained from two consecutive sessions immediately after transferring all subjects to food restriction. There was then a gap of one week to allow adjustment to the new protocol then testing was re-instated.

5.3.3 Results

Taking all the test days on food restriction there was a significant main effect between the two groups and across days. Effect of past experience ($F_{(1,72)} = 7.90$, $p = 0.014$) and of session ($F_{(6,72)} = 12.60$, $p < 0.001$) was shown by RM two-way ANOVA, there was also a

significant interaction between the two main effects ($F_{(6,72)} = 9.59$, $p < 0.001$). *Post hoc* analysis showed that the previously water-restricted subjects reached significantly lower FRs than the constantly food-restricted mice but this was only significant on sessions one though three. Since a one week non-testing gap existed between sessions 2 and 3 the data was also analysed using only the last week. Under these conditions the prior restriction protocol was lost ($F_{(1,52)} = 2.58$, $p = 0.131$ n.s.) and there was only a significant effect of session ($F_{(4,52)} = 5.51$, $p < 0.001$). *Post hoc* analysis showed that the maximum FR reached on session three was significantly lower than by session.

Figure 5.6 Maximum FR attained

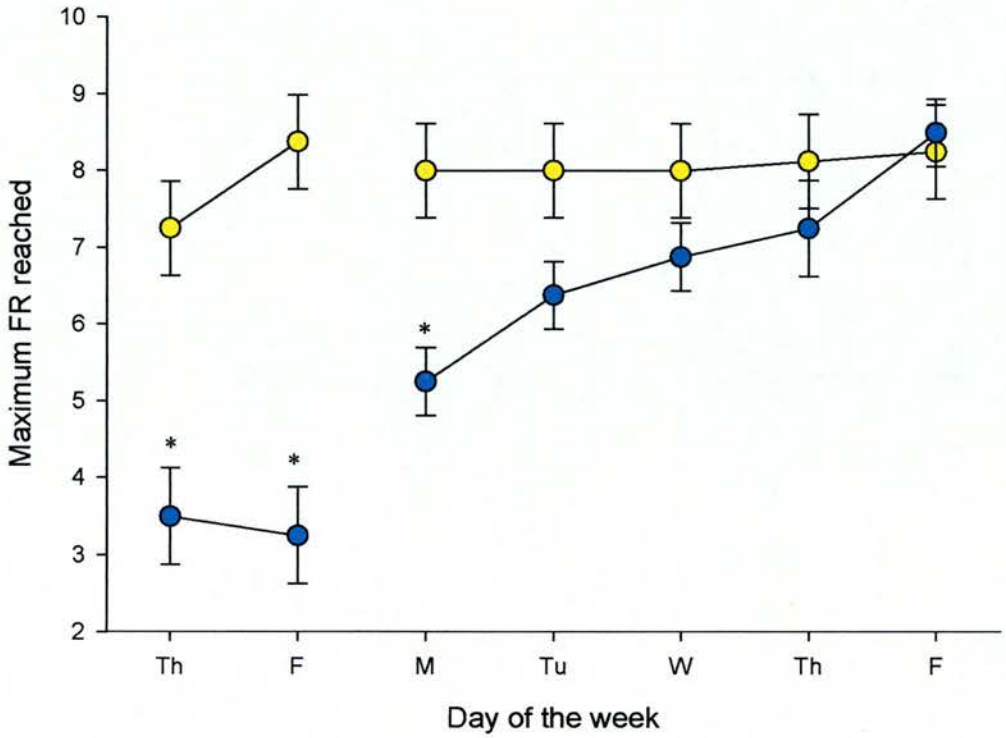


Figure 5.6 shows the maximum fixed ratio reached during the course of a session. Data is plotted as mean ± SEM by session (n = 8 per group). Break in graph indicates one week on food restriction without testing. * indicates a significant difference between the two groups, $p < 0.05$ was considered significant.

Figure 5.7 Proportionate free-feeding weight

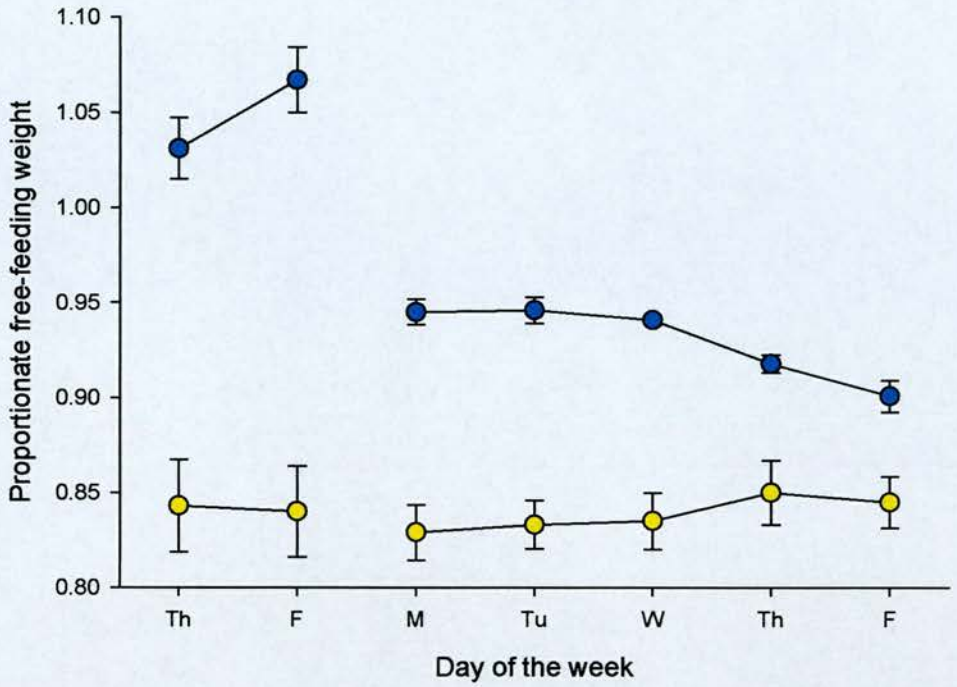


Figure 5.7 shows proportionate weights by session of the previously food-restricted (yellow symbols) and previously water-restricted (blue symbols) groups. Data is plotted as mean ± SEM of n = 8 subjects per group. Break in graph indicates one week on food restriction without testing.

5.3.4 Discussion

The data obtained from the previously water-restricted subjects were partially confounded by the timing of the task. The move from water to food restriction takes several days to be fully effective as measured by the drop in the subjects weight so over the initial 2 days under the new regime performance remained poor. Performance improved markedly after one week on the food restriction protocol with a concomitant drop in proportionate weight. The data showed that appropriately motivated animals were capable of sustaining repeated responding up to 8 or 10 nosepokes per trial to obtain reward. It is interesting to note that the subjects are driving the difficulty of the task, slow responding would still be rewarded but the fixed ratio would have remained low. Only consistently fast responding would increase the fixed ratio and therefore the perceived difficulty of the task. This protocol represents a departure from the more usual breakpoint protocols where subjects commonly receive incrementing fixed ratios independent of rate of response. Food-restricted Lister-Hooded rats have been

reported to reach 76 lever presses (equivalent to an FR 76) to obtain reward before the end of the study (Alderson, Brown et al., 2002). However their protocol used a stepwise increment of 5 presses per reward and the task only ended after either four hours or there was a five minute period without any lever presses. In contrast to reach the FR 10 noted above the mice were required to make 9 nosepokes within 6 seconds on five consecutive occasions. The protocol described here was designed to facilitate the acquisition of a specific operant response, namely perseverative responding at a single location until there was a change of state – in this case the completion of the FR and subsequent extinguishing of the cue light. It was hoped that the consolidation of this behaviour would aid mice in performing a delayed non-matching to place paradigm described later.

6 Study 5 Five choice serial response time (5CSRT) task

6.1 Introduction

The rat apparatus described by Carli, (Carli, Robbins, Evenden, and Everitt, 1983), was modified as described below. An infrared beam monitoring circuit replaced the magazine response panel (door flap) and microswitch trigger. It was anticipated that mice would find the task of pushing the magazine panel difficult or possibly even aversive. As the mice were capable of fitting into the magazine there was also a risk that they could become trapped. The reinforcement pellet delivery system described by Carli was replaced with a liquid reinforcement delivery system. This used a peristaltic pump to deliver approximately 20 µl of sweetened milk into the magazine as a single reward rather than a 45 mg reinforcement pellet. More details are given in Section 2.2.

Since mice were considered to be averse to bright lights it was also decided to also rearrange the activation of the house light. In previous rat experiments the house light remained on throughout and was only extinguished to indicate some form of error. It was decided to instead leave the house light off and activate it to indicate some kind of error, thereby linking a potentially aversive stimulus with a breakdown in correct responding. An additional benefit of this was that it would increase the contrast between a brightly lit stimulus location and the remainder of the test chamber during the session perhaps making the task easier. This also allowed the possibility of reversing the chamber conditions to increase task difficulty by having the house light on until an error was performed thus decreasing the contrast between chamber and stimulus. As noted in Section 2.2 a narrower arc of stimulus locations was used for the mice. This arc (approximately 13 cm across) was comparable to the full arc used in the smaller mouse 9 hole box designed by the then CeNeS company (personal communication) now Cambridge Cognition, Cambridgeshire. The narrower arc was used to restrict the area the mice must attend to and increase the likelihood they would acquire the task without significant location biases developing. The possibility that the stimulus arc could be broadened at a later date to increase task difficulty also remained.

For simplicity, every time a response hole or magazine beam is lit, the corresponding IR beam monitoring that aperture is also activated. Unless otherwise noted the IR beam is deactivated when the light is extinguished.

6.2 Materials and Methods: Training

6.2.1 Habituation

The initial habituation protocols are described in Section 5.2.1 and 5.2.2.

6.2.2 Simple response training.

This phase developed a cue-reward association such that a response in a lit response hole would elicit a reward at the magazine. This programme initially lit all five response holes and activated their respective IR beams. If any IR beam was broken, milk was delivered to the magazine, the response lights were extinguished and the magazine illuminated. Further activity at the now darkened response holes was non-contingent. Response at the activated magazine began a 2 second ITI during which time the magazine light was switched off. After the ITI ended the five response holes were reactivated and lit and another trial began. The programme registered the total number of trials completed but not the location of the responses. Session length was increased from 15 to 25 minutes following successful completion of 10 or more trials per session.

6.2.3 Serial response training.

In this, the final stage of the task, the response hole IR beams were constantly active irrespective of the state of the corresponding light unless otherwise stated.

Figure 6.1 indicates the schematic of the serial response task that is described in detail below. The task began by delivery of milk to the magazine accompanied by magazine activation. Nosepoke at the magazine deactivated it and began a fixed inter trial interval (standard setting of 2 sec). A nosepoke in any of the response holes ahead of the ITI completion was registered as an Anticipatory Response. Following the ITI, one of the five responses holes was lit at random. This light stimulus was left on for a period called the Stimulus Duration (SD, initially set to 10 sec). After this SD the stimulus light was extinguished but the IR beams remained active. The IR beams were only deactivated after a further delay defined as the Limited Hold (LH) duration, initially set to 2 sec. A nosepoke in the stimulus hole during the active IR beam period (SD + LH) resulted in a Correct trial and reward delivery to the magazine with attendant light and magazine IR beam activation. A response in any other hole was recorded as an Incorrect trial. A failure to make any nosepoke

during the SD + LH window was recorded as an Omission. Immediately following an Anticipatory, Incorrect or Omitted response all IR beams and stimulus lights were deactivated and the main house light was switched on for the duration of the Time out period (4 sec usually). After the time out period had elapsed the magazine was reactivated but no reward was given. In both Correct and Timed out trials the trial was completed by a nosepoke at the magazine once more. This restarted the ITI and reactivated the response IR beams. On Correct trials the ITI period was latterly extended by an additional delay called the reward duration (RD) of 4 sec. to enable the subjects to consume their reward without missing further trials. The latency to make a correct response was also recorded as the time taken to respond after stimulus presentation.

The software registered all responses, latencies and number of stimuli presented by response hole location. The number of omitted trials for any one hole was subsequently calculable. Accuracy was based upon the number of responses made and expressed as the proportion correct as given below:

Equation 6.1

$$\text{Accuracy} = \frac{\text{Correct Responses}}{\text{Total Correct Responses} + \text{Total Incorrect Responses}}$$

Initial stimulus duration (SD) for the 5CSRT task was set to 10 sec, with an attendant 2 sec limited hold (LH). Criterion for reducing SD was attaining a mean correct latency below 50% of the current SD from a minimum of 10 correct responses. SD was reduced from 10 sec to 8 sec initially. Following this the SD was reduced to 4, 2 and 1 sec upon re-attaining the criterion at the shorter durations. LH was reduced from 2 sec to 1 sec once the SD reached 4 sec.

Figure 6.1 Single trial progression in 5CSRT task.

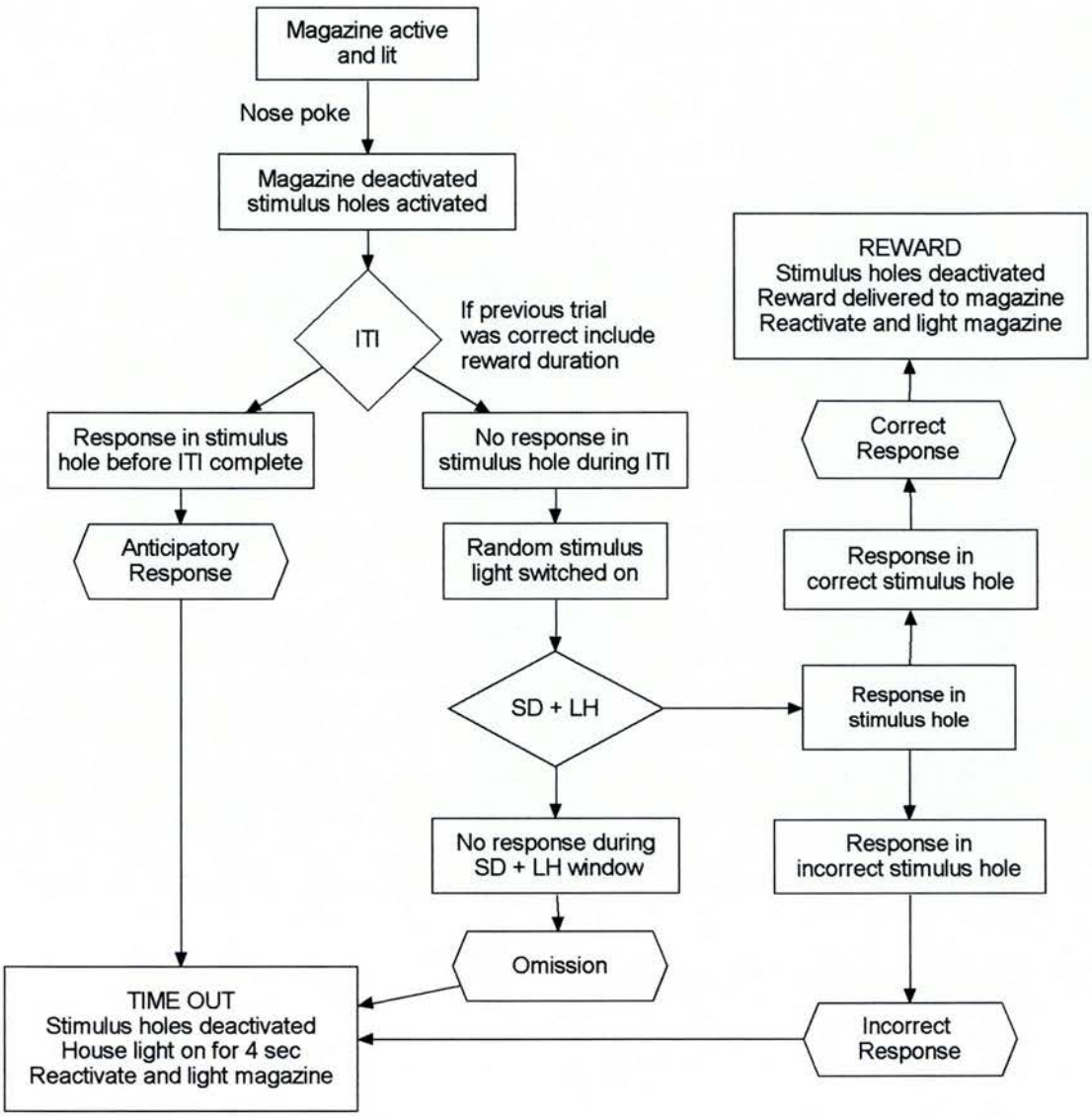


Figure 6.1 shows the responses and outcomes possible within a single trial in the 5CSRT task. Diamonds indicate within-trial timer activations. Hexagons indicate catalogued actions by the subject on which performance is measured. Rectangles indicate programmed consequences and decisions by the subject. ITI, inter trial interval; SD, stimulus duration; LH, limited hold. Trial ends with either TIME OUT or REWARD which returns programme to 'Magazine active and lit'.

6.3 Experimental set-up

Groups of eight C57Bl/6J and 129P2/Ola mice were used in this study. The protocol used is given above with slight modifications for the poorest performing animals. In the early stages of training if mice were performing poorly they were run on the previous stage of training

immediately before the more difficult session i.e. running mice on the simple response task for 15 min prior to the final 5CSRT task. Following attainment of a final performance criterion of 120 trials completed in less than 25 minutes, with >80 correct and <20 % omissions the mice were subjected to a series of psychophysical challenges.

Previously all appetitive rat behaviour carried out here used solid food reinforcement. It was of interest to determine whether rats would learn a task for sweetened milk solution. Since the vast bulk of the literature using the 5CSRT task including most of the pharmacology and lesion studies have been done in various strains of rat it was also necessary to use rats in our modified variant to bridge the gap between species and task modifications. The introduction of an infrared magazine monitor rather than the tactile stimulus of the original door flap might have altered performance. Therefore in a separate experiment twenty four male Lister Hooded rats were also ran on this protocol. The data from the various challenges was analysed together to determine whether differences in strain performance existed. The rat experiment adhered to the protocol defined above with the exception that they did were maintained on a 0.25 sec stimulus duration baseline before undergoing the challenges.

6.3.1 Stimulus duration challenge

In order to probe the ability of the subjects to perform under more demanding circumstances the stimulus duration was reduced. To assess the possibility of improvement through familiarity to the increased difficulty levels the subjects were tested on 3 consecutive days at each probe SD. In the mice each three day probe period was separated from the next probe period by a baseline day at the standard 1 sec SD + 1 sec LH. The subjects were challenged with 0.75, 0.5 and 0.25 sec SD on consecutive weeks with a corresponding LH of 0.75, 0.5 or 1.0 sec respectively. Because the rats rapidly attained good performance at the 1 sec stimulus duration the baseline was reduced to 0.25 sec. In this case the 1 sec stimulus duration data was taken from the final three sessions at that duration.

6.3.2 Stimulus intensity challenge

Following the SD challenge a new baseline was set with 1 sec SD (0.25 sec for the rats) and 0.5 sec LH. This ran for two days prior to the stimulus intensity challenge. Stimulus detection by the mice was then examined by reducing the intensity of the stimulus lights. Stimulus intensity was measured by placing the light meter (Model RS180-7133, RS

Components Ltd., UK) with the detector 50 cm from the centre of the stimulus array. A single stimulus light was activated with no other light present in the room. The three levels of intensity were then defined and measured in all test chambers. Normal (high intensity) level was measured at a mean of 97.5 lux, Medium level was 21.25 lux and Low level was 1 lux. In this challenge the animals only received one session at each of the three levels using a randomised order of intensity presentation.

6.3.3 Random ITI challenge

The final psychophysical challenge was to disrupt the anticipated timing of the task. All subjects received a one full session at each of the following ITI: 2, 4 and 8 seconds. The order of ITI presentation was randomised across the group over three consecutive days. The normal ITI of 2 sec was included as control values. At this stage one C57 and one 129P2 mouse was withdrawn from testing due to ill health.

6.3.4 Noise distractor challenge

Another form of visual distractor in this task is the presentation of a novel stimulus as noise to which subjects must respond differentially than they would to a normal trial. To investigate the effect of a novel noise stimulus on performance a separate programme was written. The standard 5CSRT task created a list of five possibilities (the five stimulus locations) and then randomising it to create a unique sequence of signal presentations each session. The new programme incorporated a sixth possibility with equal weighting into the randomised list, thus creating the six choice serial response task (6CSR task). The sixth possibility did not have a stimulus light output slaved to it so when it appeared in the random sequence no stimuli appeared. This was called a noise trial, during a noise trial a response in any hole was classed as an Incorrect Response or False Alarm resulting in the usual Time out. Making no response on a noise trial was categorised as a Correct Rejection leading to reward. Performance in this challenge was measured by standard accuracy of appropriate responding to the lit signal stimuli (see Section 6.2.3). Inappropriate responses on the noise trials were included in this standard accuracy measure like the other incorrect responses. Accuracy of appropriate omissions (correct rejections) to the noise trials were handled with a separate accuracy measure:

Equation 6.2

$$\text{Correct Rejection Accuracy} = \frac{\text{Number of correct rejections}}{\text{Number of noise trials presented}}$$

The subjects were given three consecutive days on the 6CSR task flanked by one baseline day on the standard 5CSRT task either side to investigate whether performance was perturbed by the challenge.

6.3.5 *Ad. lib.* feeding challenge

The previous chapter indicated that level of motivation affected performance of a simple operant task. It was of interest to determine what effects *ad. lib.* feeding would have on the various indices of performance used in the 5CSRT task. The motivational challenge involved placing the subjects on *ad. lib.* feeding for 18 hours prior to a final session at standard baseline parameters was carried out. Performance in this was compared to two previous days on identical test parameters whilst restricted feeding was maintained. This challenge was not carried out on the rats, only a mouse strain comparison was possible.

6.4 Results

6.4.1 Acquisition

Inspection of the data during the acquisition phase of the study indicated that the 129P2 group acquired the task more readily than the C57 group. This was indicated by a consistently higher accuracy throughout acquisition and reaching asymptotic performance earlier in training. As this was not intended as a pure acquisition experiment the different groups were not treated equally, some subjects received additional training where it seemed prudent. Unfortunately due to this flexible approach to training it was not considered appropriate to statistically quantify any differences. The rats also acquired the task quickly and reached the shorter stimulus durations faster than the mice, however their performance decreased when placed on the 0.25 sec baseline (Figure 6.2). The rats showed a twofold higher anticipatory response rate than either mouse strain early in training. However, their anticipation levels rapidly decreased during acquisition (Figure 6.4).

Figure 6.2 Accuracy during acquisition

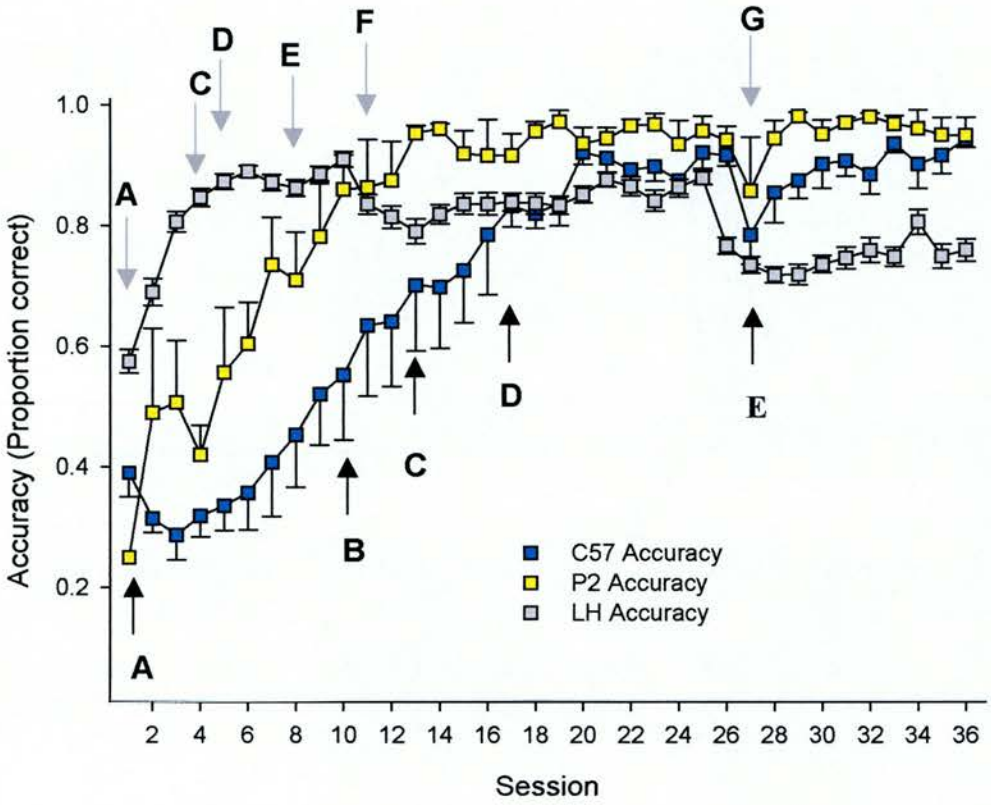


Figure 6.2 shows the mean accuracy by session during acquisition of the 5CSRT task. Data is plotted as mean ± SEM of 8 mice per strain or 24 rats. Mean stimulus duration was reduced at the points indicated by black arrows for the mice and grey arrows for the rats. Stimulus duration was as follows: A:10, B:8, C:4, D:2, E:1, F:0.5 and G:0.25 sec.

Figure 6.3 Total trials throughout acquisition

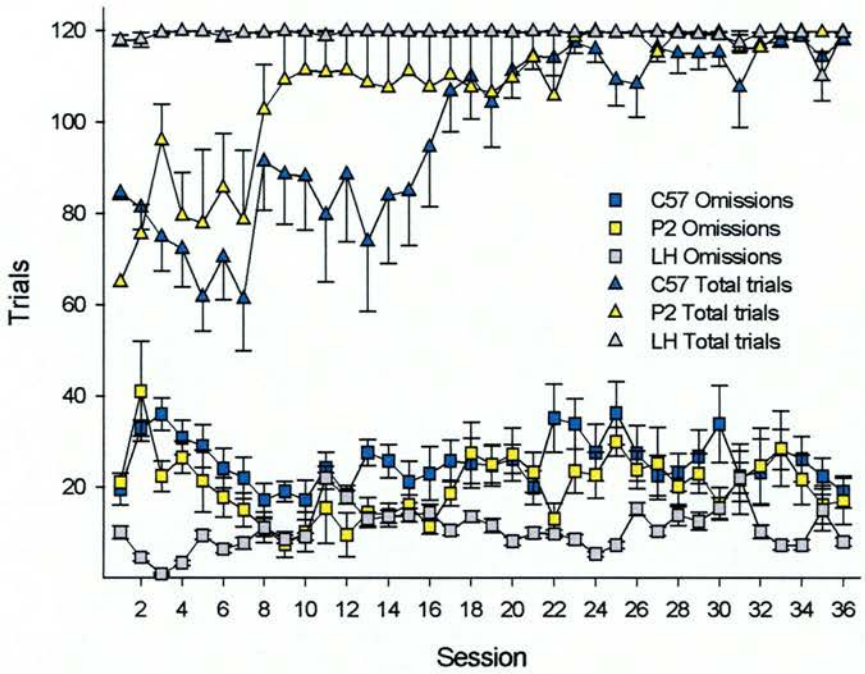


Figure 6.3 shows number of trials omitted (square symbols) and total number of trials completed (triangle symbols) by session during acquisition of the 5CSRT task. Data is plotted as group mean ± SEM from 8 mice and 24 rats per group.

Figure 6.4 Anticipations through acquisition

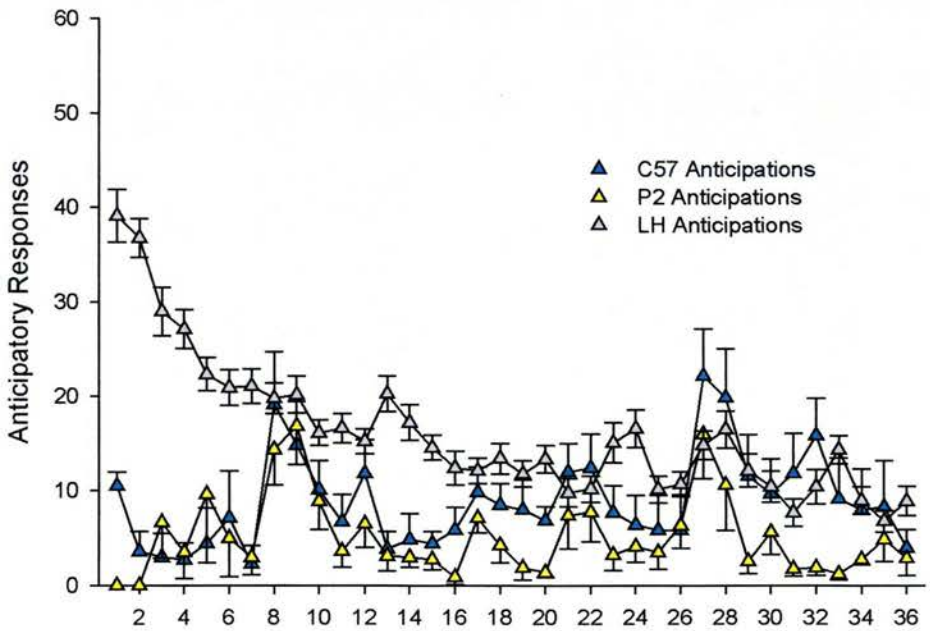


Figure 6.4 shows number of anticipatory responses by session during acquisition. Data is plotted as group mean ± SEM from 8 mice and 24 rats per group.

6.4.2 Stimulus duration challenge

To determine the effects of repeated exposure to the stimulus duration challenges the data was analysed initially separately by strain. Shortening the stimulus duration resulted in a duration-dependent reduction in accuracy within each strain. For the C57 strain there was no significant effect of repeating the SD challenge over three consecutive days on accuracy, effect of repeat ($F_{(2,24)} = 2.182$, $p = 0.155$ n.s.). Within the 129P2 strain there was a significant effect of repeat and also a significant interaction with stimulus duration, effect of repeat ($F_{(2,28)} = 27.53$, $p < 0.001$) and effect of interaction ($F_{(4,28)} = 8.74$, $p < 0.001$). *Post hoc* analysis showed that there was no effect of repeated challenge at either the 1 sec or 0.5 sec stimulus duration challenges. However at the 0.25 sec SD accuracy did change with repeated exposure. However, accuracy actually dropped from 94 % to 70 % accuracy rather than improved as was hypothesised. Within the LH rat group there was also a significant effect of repeat and an interaction with duration on accuracy, ($F_{(2,91)} = 11.77$, $p < 0.001$) and ($F_{(4,91)} = 2.76$, $p < 0.032$) respectively. In this case *post hoc* analysis did not show any effect of repetition at the 0.25 sec SD, which showed that the rats baseline accuracy was stable. However it did reveal an effect of improvement in accuracy at the 1 sec SD between the first and third day challenge (86 % to 91 % accuracy). However since this data was taken from early in the rats' learning curve such an improvement was to be expected. Because the statistical software available was unable to manage three way repeated measures ANOVA which would have allowed for a Strain X Stimulus Duration Treatment X Repeat analysis it was decided to collapse the data across days. It was recognised that by collapsing the individual subject's data over the three days within each stimulus duration some sensitivity would be lost in this challenge. However it was felt that more would be gained by using repeated measures analysis on two factors (Strain X Treatment) rather than three factor analysis without repeated measures. Furthermore, the evidence for experience-based changes in performance seemed to be limited to the early part of the learning curve in rats and the very short stimulus duration in mice.

Two way RM ANOVA showed an effect of stimulus duration (SD) ($F_{(2,72)} = 57.374$, $p < 0.001$) but not of strain ($F_{(2,72)} = 2.295$, $p = 0.115$ n.s.) on accuracy. Tukey's *post hoc* analysis demonstrated that accuracy was significantly lower at the 0.25 sec SD (Figure 6.5). Analysis of the logged omissions data showed significant effects of strain ($F_{(2,72)} = 76.743$, $p < 0.001$) and SD ($F_{(2,72)} = 10.75$, $p < 0.001$). Again a significant interaction was seen ($F_{(4,72)} = 23.463$, $p < 0.001$). *Post hoc* analysis showed that the LH rats made significantly fewer

omissions than either mouse strain at the 0.5 and 0.25 sec SD but that overall omission levels rose as SD decreased. The latency to respond correctly was significantly altered independent of strain, main effect of SD ($F_{(2,72)} = 262.721$, $p < 0.001$) using logged data. *Post hoc* comparison showed that the groups responded correctly significantly faster at the shorter stimulus durations than at all longer durations. This result implies they were performing better at the shorter durations however this effect is balanced against the rise in omissions and reduction in responded trials at the shorter durations (data not shown). Analysis of the number of anticipatory responses made demonstrated both strain and stimulus duration effects ($F_{(2,72)} = 11.509$, $p < 0.001$) and ($F_{(2,72)} = 6.363$, $p = 0.003$) respectively. A significant interaction was also seen ($F_{(4,72)} = 8.475$, $p < 0.001$). *Post hoc* analysis showed that the LH rats made significantly more anticipations than either mouse strain during this challenge. Overall anticipations were highest at the 1 sec SD and appear to decrease at the shorter SD challenges. However the rats were last placed on the 1 sec SD early in training (see Figure 6.2) and so the elevated anticipations at that SD are likely to be due to still learning the task rather than an effect of the challenge.

Figure 6.5 Effects of stimulus duration challenge

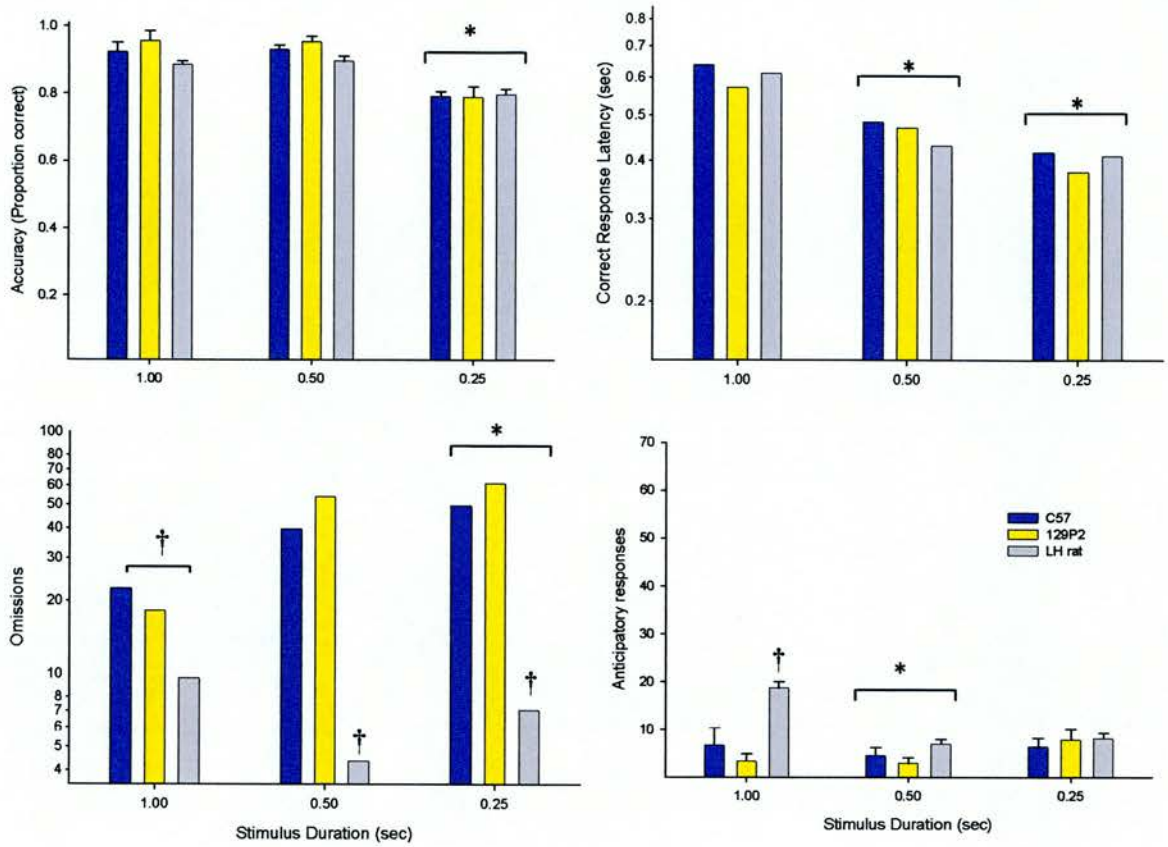


Figure 6.5 shows accuracy (top left panel), correct response latency (top right panel) omissions (bottom left panel) and anticipatory responses (bottom right panel) during the stimulus duration challenge. Data are plotted as mean ± SEM by strain for C57 (blue, n=7), 129P2 (yellow, n=8) and LH rat (grey, n=24). * indicates significant difference from 1 sec stimulus duration (control condition for mice). † indicates significant difference from other groups at the that challenge. $p<0.05$ was significant.

6.4.3 Stimulus intensity challenge

Accuracy was significantly altered at reduced stimulus intensity (SI). There was both a main effect of strain ($F_{(2,72)} = 45.108$, $p < 0.001$) and of SI ($F_{(2,72)} = 225.368$, $p < 0.001$). There was also a significant interaction ($F_{(4,72)} = 14.678$, $p < 0.001$) between the main effects. *Post hoc* analysis showed that accuracy was significantly reduced at the lowest intensity (1 lux) in all strains (Figure 6.6). Furthermore, the LH rats were significantly less accurate at all SI values than either mouse strain. A dissociation between mouse strain performance was only seen at the lowest SI, 1 lux, where the 129P2 strain were significantly more impaired than the C57 strain. Omission levels rose significantly as stimulus intensity was decreased using log transformed data. There was a significant effect of strain ($F_{(2,72)} = 19.998$, $p < 0.001$) and SI ($F_{(2,72)} = 51.996$, $p < 0.001$). *Post hoc* analysis showed omission levels at the lowest intensity to be significantly higher than at either of the other two SI levels. Furthermore, the LH rats made significantly fewer omissions than either mouse strain at both 100 lux and 20 lux conditions. Correct response latency also showed significant strain and SI effects ($F_{(2,72)} = 23.511$, $p < 0.001$) and ($F_{(2,72)} = 55.811$, $p < 0.001$) respectively using logged data. A significant interaction was also seen ($F_{(4,72)} = 4.981$, $p = 0.001$). *Post hoc* analysis showed that at the lowest intensity, correct response latency was significantly longer than during either of the higher intensity conditions. Again the LH rats responded significantly faster than either mouse strain at the higher SI conditions. Anticipatory responses were significantly altered by reducing the stimulus intensity. There were main effects of strain ($F_{(2,72)} = 8.662$, $p < 0.001$) and SI ($F_{(2,72)} = 21.377$, $p < 0.001$). There were no significant interactions between these effects ($F_{(4,72)} = 1.086$, $p = 0.37$ n.s.). *Post hoc* analysis showed that the LH rat group committed significantly more anticipatory responses than either mouse strain overall. At the lowest stimulus intensity (1 lux) all groups showed an increase in anticipatory responses.

Figure 6.6 Effects of stimulus intensity challenge

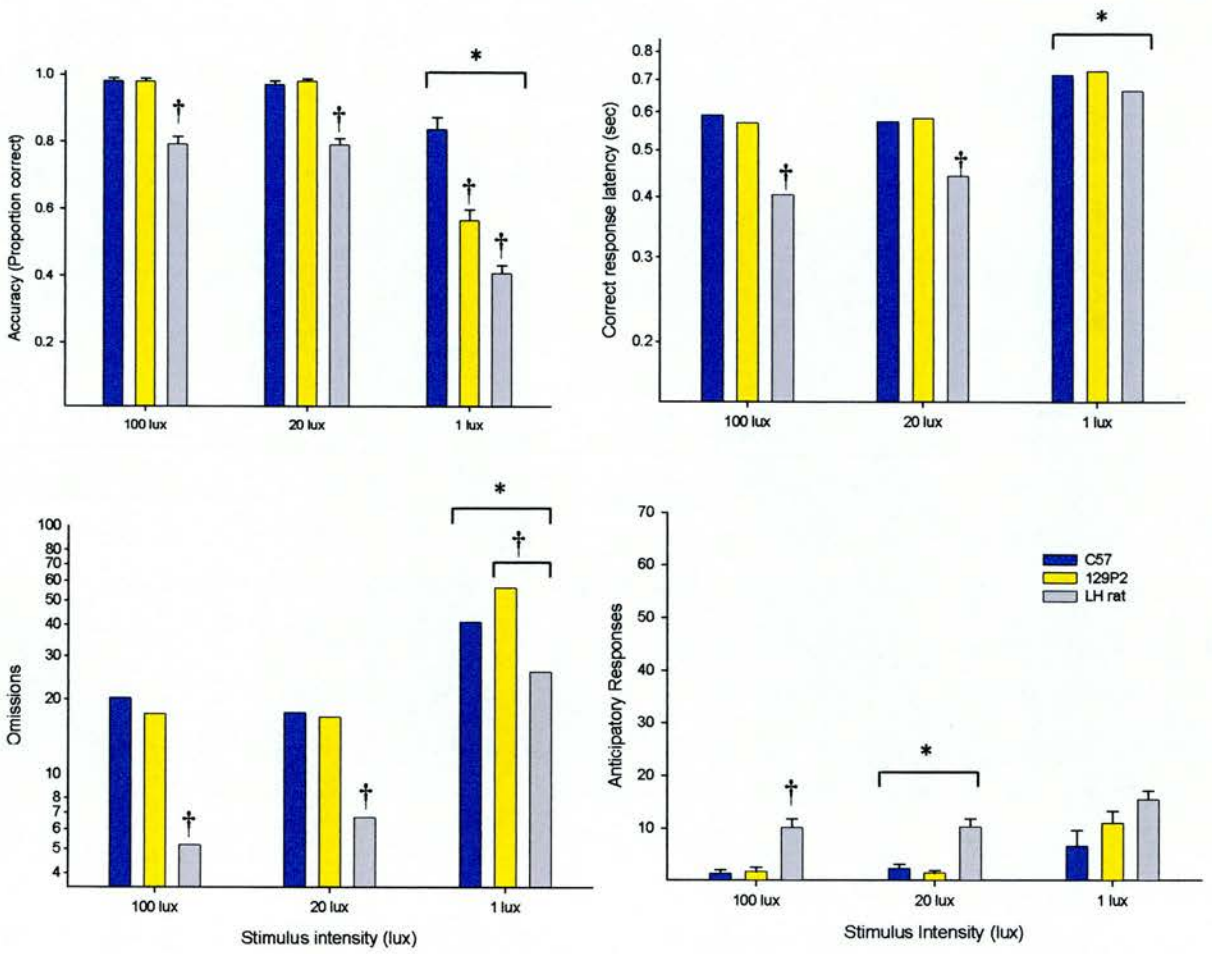


Figure 6.6 shows accuracy (top left panel), correct response latency (top right panel) omissions (bottom left panel) and anticipatory responses (bottom right panel) during the stimulus intensity challenge. Data are plotted as mean ± SEM by strain for C57 (blue, n=7), 129P2 (yellow, n=8) and LH rat (grey, n=24). * indicates significant difference from 1 sec stimulus duration (control condition for mice). † indicates significant difference from other groups at the that challenge. $p<0.05$ was significant.

6.4.4 Random ITI challenge

By imposing a longer ITI a significant shift in accuracy was seen, effect of strain ($F_{(2,67)}$ = 107.335, $p < 0.001$) and effect of interval ($F_{(2,67)} = 7.826$, $p < 0.001$). A significant interaction between these main effects was also seen ($F_{(4,67)} = 3.364$, $p = 0.014$). *Post hoc* analysis showed that accuracy fell significantly as ITI was increased and that the LH rats were significantly less accurate than the mice throughout (Figure 6.7). These effects were almost exclusively due to the decline in performance in the LH rats at the longer ITI's. The LH group became significantly less accurate as the ITI increased whereas accuracy in both mouse strains was unimpaired by this challenge. Omission levels were also affected by increasing the ITI, main effect of strain ($F_{(2,68)} = 9.428$, $p < 0.001$) and main effect of interval ($F_{(2,68)} = 14.173$, $p < 0.001$) using logged data. There was also a significant interaction between the two main effects ($F_{(4,68)} = 6.884$, $p < 0.001$). *Post hoc* analysis demonstrated that overall omission levels rose significantly by increasing the ITI to 8 sec. Within strain there was no increase in omission levels for the two mice strains but an increase in omissions made by the LH rats. This increase was significant both at the 4 sec and 8 sec ITI challenges. Elevating the ITI caused a similar change in correct response latency, effect of strain ($F_{(2,67)}$ = 37.648, $p < 0.001$) and effect of interval ($F_{(2,67)} = 19.699$, $p < 0.001$) using logged data. An interaction was also seen between these effects ($F_{(4,67)} = 4.092$, $p = 0.005$). *Post hoc* analysis showed that correct latency during the 8 sec ITI challenge was significantly longer than at the shorter intervals. Within strain the 129P2 mice were unaffected by changing the ITI, whereas both the C57 and LH strains were significantly slower at the 8 sec ITI than at either 2 or 4 sec ITI. The LH rats remained faster than either mouse strain throughout the challenge. The strongest effects of this challenge were on the level of anticipatory responding. There were significant main effects of strain and interval ($F_{(2,68)} = 23.703$, $p <$ 0.001) and ($F_{(2,68)} = 63.735$, $p < 0.001$) respectively. A significant interaction between these main effects was also seen ($F_{(4,68)} = 10.451$, $p < 0.001$). *Post hoc* analysis showed that the level of anticipatory responding elevated as the ITI was increased. Again the LH rats committed significantly more anticipatory responses than either mouse strain throughout. A dissociation was seen between the mouse strains with the C57 strain making significantly more anticipations at the 4 sec ITI challenge whereas the 129P2 were only affected by the 8 sec ITI.

Figure 6.7 Effects of inter trial interval challenge

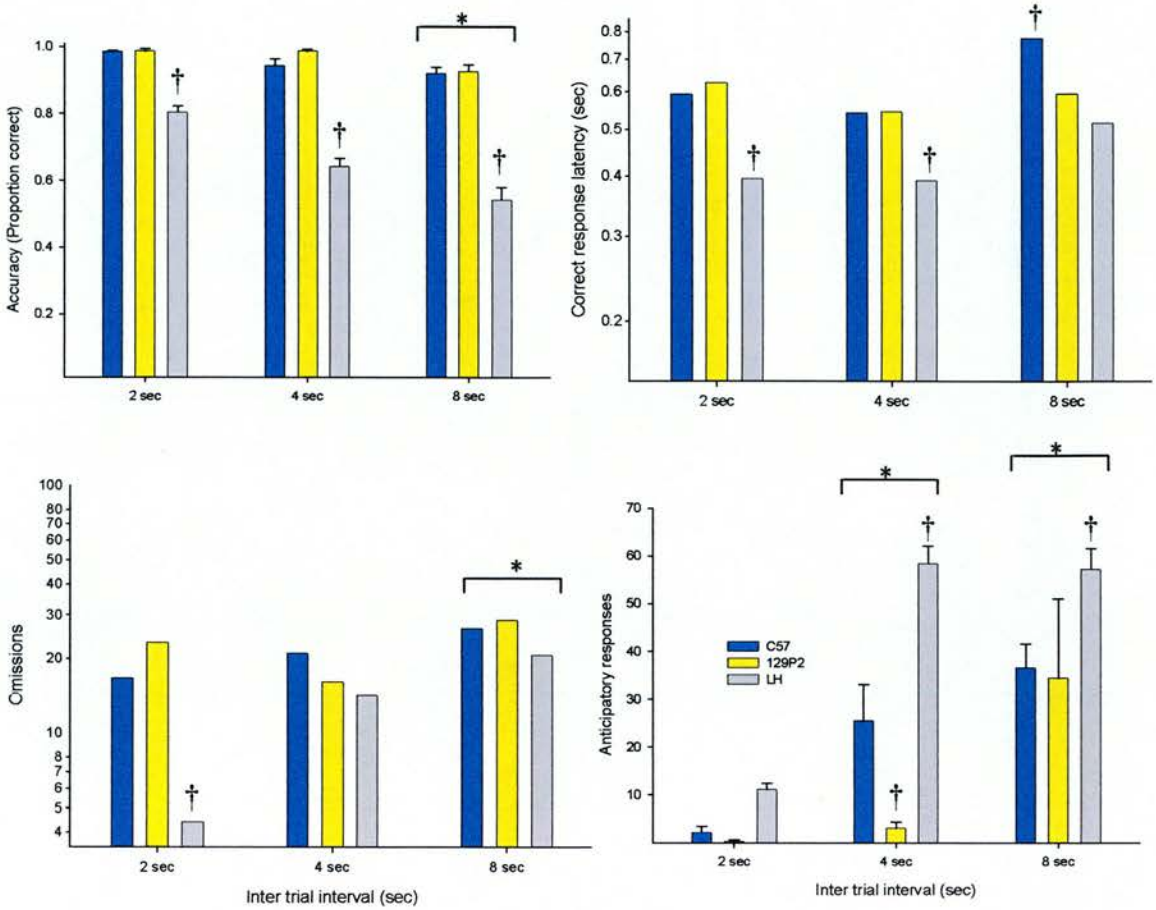


Figure 6.7 shows accuracy (top left panel), correct response latency (top right panel) omissions (bottom left panel) and anticipatory responses (bottom right panel) during the inter trial interval challenge. Data are plotted as mean ± SEM by strain for C57 (blue, n=6), 129P2 (yellow, n=7) and LH rat (grey, n=24). * indicates significant difference from 2 sec inter trial interval (control condition for both mice and rats). † indicates significant difference from other groups at the that challenge. $p<0.05$ was significant.

6.4.5 Noise distractor challenge

Response accuracy was significantly altered by strain and by day ($F_{(2,140)} = 20.112$, $p < 0.001$) and ($F_{(4,140)} = 5.147$, $p < 0.001$) respectively. No significant strain vs. day interaction was seen ($F_{(8,140)} = 1.815$, $p = 0.079$, n.s.). *Post hoc* analysis showed that accuracy significantly improved after noise trial withdrawal (Baseline 2) compared to the initial

exposures to noise trial (D1 and D2). However, there were no significant effects on performance between the initial baseline (Baseline 1) and any of the noise trial sessions (Figure 6.8). The LH rats maintained a poorer level of performance than the mice throughout. The accuracy of appropriate omission to a noise trial presentation was assessed as correct rejections (see Section 6.3.4 for details), defined as the proportion of noise trials not responded to. Again there was a significant strain effect ($F_{(2,72)} = 29.348$, $p < 0.001$) based around a significantly poorer correct rejection rate in the LH rat strain. However there were no significant effects of prior exposure, effect of day ($F_{(2,72)} = 0.659$, $p = 0.573$, n.s.). Mean correct rejection accuracy in the mice was only slightly lower than the standard accuracy measure (C57 84.2 % correct rejection accuracy vs. 93.3 % accuracy; 129P2 88.8 % correct rejection accuracy vs. 95.7 % accuracy). The slight discrepancy between the Correct Rejection accuracy and Accuracy values may explain the rise in incorrect response levels in the noise trial sessions as noted below. In contrast, LH rats showed a massive discrepancy between standard accuracy and correct rejection rate (48.4 % correct rejection accuracy vs. 78.2 % accuracy) indicating a much poorer restraint on the part of the subjects during noise trials.

Figure 6.8 Accuracy and correct rejection accuracy

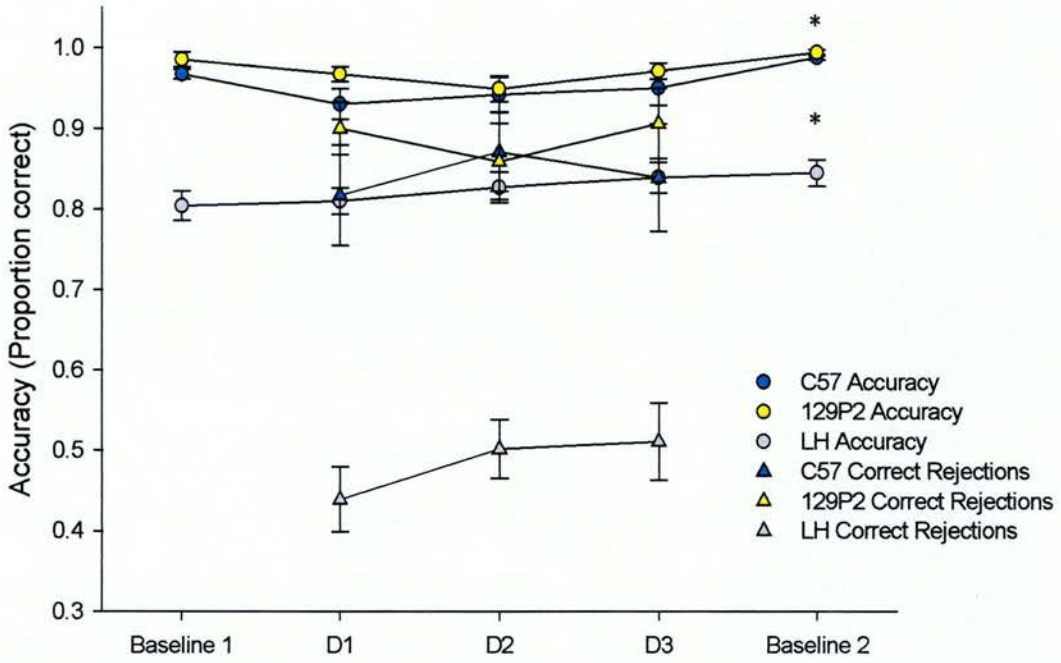


Figure 6.8 shows group accuracy (mean ± SEM) of the three strains over the three noise distractor challenge sessions and the two flanking baseline sessions. Circles indicate accuracy of appropriate responding to the standard lit stimuli. Triangles indicate correct rejection performance i.e. accuracy of 'appropriate failure to respond' to the noise challenge. * indicates significant difference from Baseline 1, $p < 0.05$ was significant.

Incorrect responses were not significantly altered by the noise trial sessions, effect of day ($F_{(4,140)} = 2.421$, $p = 0.051$ n.s.). However there was a significant strain effect ($F_{(2,140)} = 24.282$, $p < 0.001$) with the LH rats making significant more incorrect responses than either mouse strain throughout (Figure 6.9). Inspection of the data showed that incorrect response levels did rise slightly in both mouse strains during the noise trial sessions, but any effect of this rise was masked by the threefold difference in rat vs. mouse incorrect response levels.

Figure 6.9 Incorrect responses

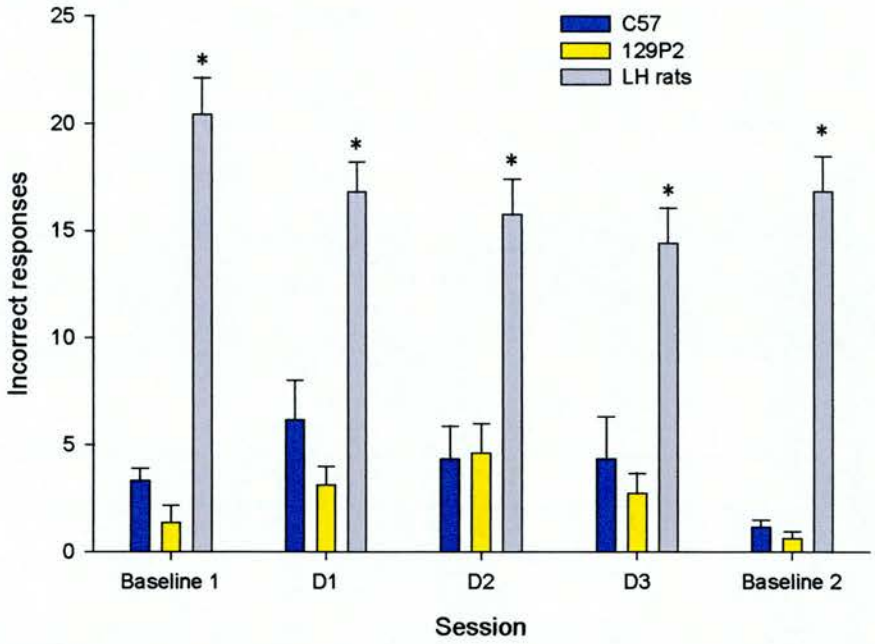


Figure 6.9 shows mean ± SEM incorrect responses during noise trial challenge. * indicates significant difference from other strains on that session. $p < 0.05$ was significant.

Mean correct response latency was also altered by exposure to the noise challenge, effect of strain ($F_{(2,140)} = 36.814$, $p < 0.001$) and effect of day ($F_{(4,140)} = 8.298$, $p < 0.001$). Inspection of the data showed that the LH rats responded significantly faster than either mouse strain overall (Figure 6.10). There was also a slight increase in response latency after noise withdrawal in the mice only.

Figure 6.10 Correct response latency

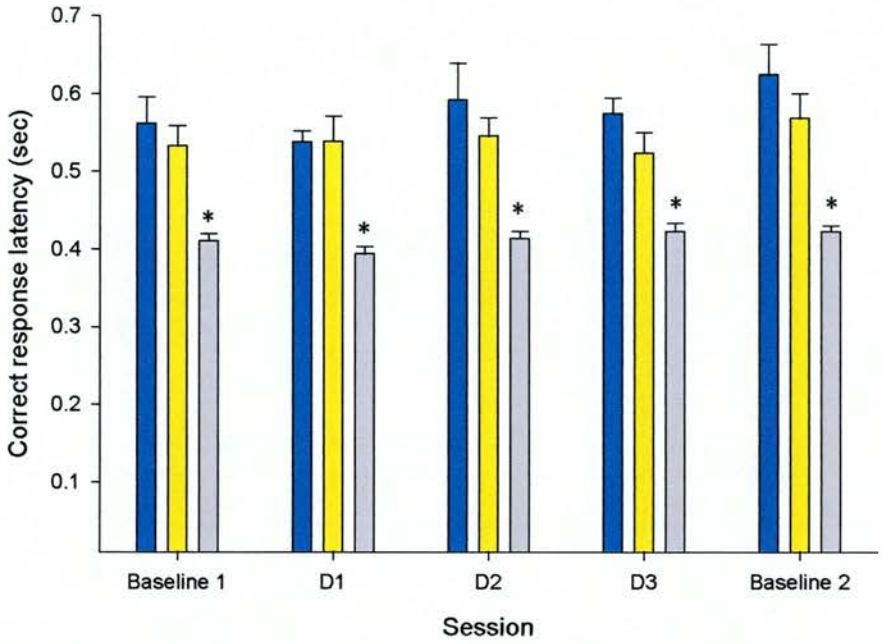


Figure 6.10 shows correct response latency (latency does not include noise trial presentations). Mean ± SEM is plotted, * indicates significant difference from other strains at on that session, $p < 0.05$ was significant.

6.4.6 *Ad. lib.* feeding challenge

A single 18 hour period of free access to food and water significantly affected performance in a strain independent manner in comparison to performance immediately before free-feeding period. Feeding state significantly affected omissions ($F_{(1,11)} = 20.009$, $p < 0.001$) with *post hoc* analysis showing omissions significantly above baseline following free-feeding (Figure 6.12). A similar effect was seen on correct latency ($F_{(1,11)} = 15.484$, $p = 0.002$) with latency to respond increasing after *ad. lib.* feeding (Figure 6.13). Conversely, accuracy was positively affected by *ad. lib.* feeding, effect of feeding state ($F_{(1,11)} = 6.649$, $p = 0.026$). Further analysis showed that accuracy was significantly improved by *ad. lib.* feeding (Figure 6.11).

Figure 6.11 Accuracy

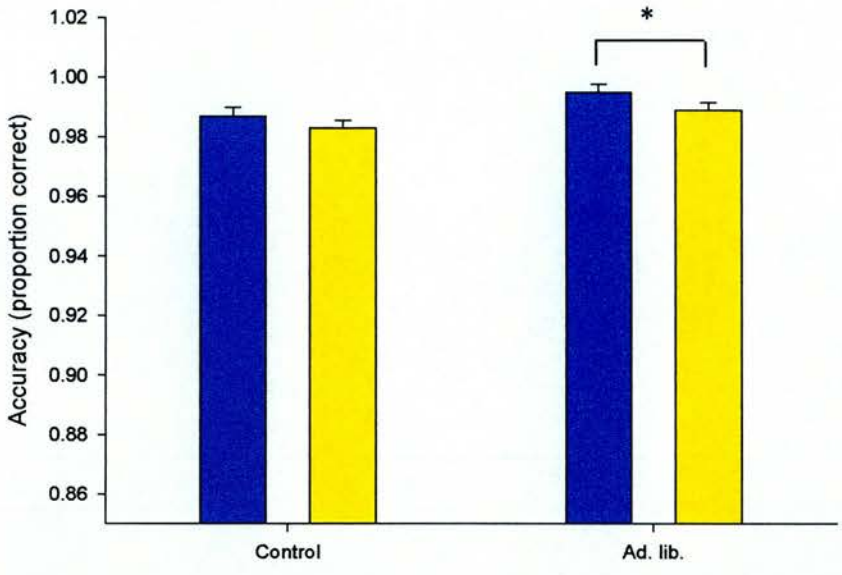


Figure 6.11 shows accuracy of responses before and after 18 hours *ad. lib.* feeding. Data is plotted as group mean ± SEM proportion correct responses. * indicates significant difference from control values, $p < 0.05$ was significant.

Figure 6.12 Omissions

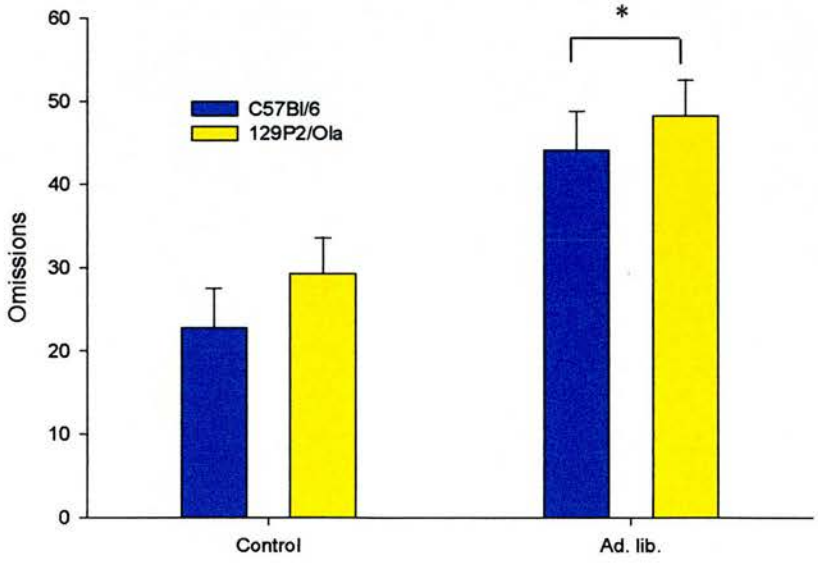


Figure 6.12 shows omission levels before and after 18 hours *ad. lib.* feeding. Data is plotted as group mean ± SEM. * indicates significant difference from control values, $p < 0.05$ was considered significant

Figure 6.13 Correct latency

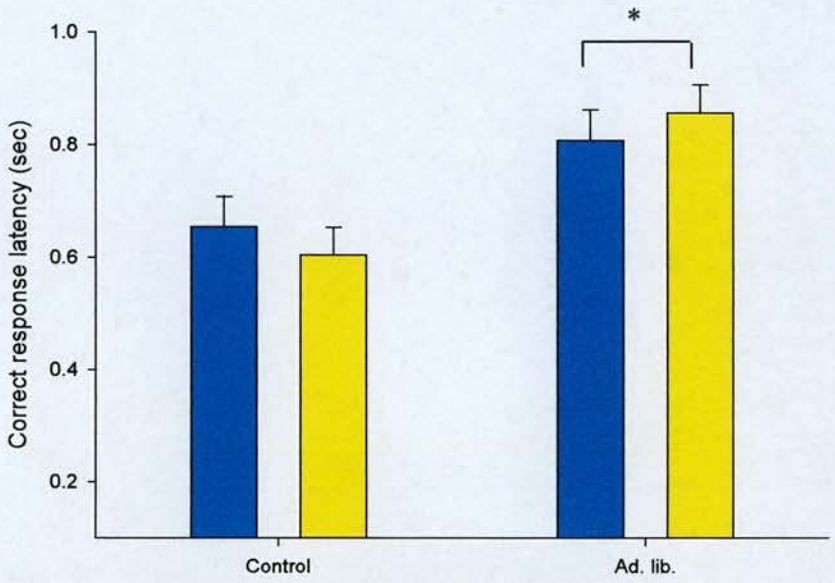


Figure 6.13 shows correct response latency before and after 18 hours *ad. lib.* feeding Data is plotted as group mean ± SEM. * indicates significant difference from control values, $p < 0.05$ was significant.

6.5 Discussion

The pilot studies used solid reinforcement due to equipment availability. A correct response was rewarded with a 45 mg reinforcement pellet (P.J. Noyes Co. Ltd., New Hampshire, USA). This was previously a standard approach in rats and well-trained rats would obtain upwards of 100 pellets in a session. However, the mice were apparently rapidly sated when being rewarded with pellets, with instances of mice continuing to carry out the task correctly without consuming all their rewards. An initial modification to the protocol imposed a ratio of correct responding onto the programme to reduce the density of reward. This forced the subject to complete a set number of trials correctly in order to be rewarded once, up to three correct trials were required to be rewarded once. This approach did result in an increase in the total trials completed and the number of correct trials. However it was still deemed necessary to move to a liquid reinforcement schedule

Using a liquid-reinforced schedule both mice and rats were capable of learning the 5CSRT task although there did appear to be slight differences in the rate of acquisition with the C57 group acquiring the task slowest. Overall the rats made significantly fewer omissions throughout and were faster at making correct responses although their accuracy tended to be lower than that of the mice. This latter difference can be accounted for by the much shorter stimulus duration (0.25 sec rather than 1 sec SD) the rats were maintained on. Particularly noteworthy is the observation that the rats' mean correct response latency did not fall below 0.35 sec. This means the majority of their correct responses were being carried out after the stimulus duration was completed and the stimulus light was extinguished, they were responding during the limited hold. In comparison the mice rarely made correct responses slower than the current SD except at the 0.25 sec challenge. Observations showed the mice commonly ceased moving towards the stimulus location once the light went out. This suggests they were conditioned to the light cue rather than the stimulus location whereas the rats continued towards the stimulus location. In our data the rats' response latency was only slightly higher than the SD so the effect could purely be ballistic. However there is a suggestion that the rats are encoding the spatial location of the stimulus in working memory. Mizra et al (Mirza and Stolerman, 1998) challenged LH rats with a decreasing SD (1 sec down to 0.25 sec). Accuracy dropped from 82 % to 53 % but their response latency increased from 0.85 sec to 1.2 sec. It is possible the increase in latency is due to the need for a recall process. Although accuracy does drop it was still far above the 20 % chance response accuracy. In the presence of the lit stimulus the subject only requires to move towards the light, however once the stimulus is extinguished they must remember its location. Had they used the SD to orientate themselves appropriately you might expect latency to stay the same. That it increased suggests at least hesitancy but it may indicate some memory recall process.

Decreasing stimulus duration only impeded accuracy at the lowest stimulus duration, independent of strain. A strain-dependent increase in omissions was also seen in the mice with decreasing stimulus duration. There was a corresponding decrease in correct response latency. As noted before a reduction in correct response latency can be taken as an improvement in performance but this contrasts with the two to threefold increase in omissions in the mice. The rats were not adversely affected by the stimulus duration challenge. This is likely to be due to their extensive exposure to the 0.25sec stimulus duration as their baseline condition. If anything the LH rats performed worse at the 1 sec SD because that data was early in their acquisition (see Figure 6.2). It is therefore difficult to draw comparisons between mice and rats for this challenge.

Decreasing stimulus intensity from 100 lux to 20 lux had no apparent effects on any parameter but at 1 lux intensity there was a significant decrease in performance on all indices measured. Furthermore, there was a strain dissociation in task accuracy at the 1 lux stimulus intensity with the 129P2 group being significantly impaired in comparison with the C57 group. The LH group showed poorer accuracy than either strain at all intensities. All groups showed an increase in anticipatory responses at the 1 lux stimulus intensity.

Increasing the inter trial interval (ITI) showed a decrease in accuracy only at the 8 sec ITI. However this was due exclusively to the declining performance in the LH group as ITI increased. The LH strain also made significantly more omissions at the 4 sec ITI than at the 2 sec ITI but overall, omission levels only rose at the 8 sec ITI. Correct response latency also increased significantly at the 8 sec ITI. This also showed a strain dissociation with the LH and C57 strains being significantly slower at 8 sec ITI than at the shorter ITI's whereas the 129P2 strain were unperturbed on this parameter. The number of anticipatory responses was substantially perturbed by increasing the ITI. The number of anticipations rose as the ITI increased. This rise was significant for C57 and LH groups at 4 sec but only significant at 8 sec for the 129P2 group.

With the exception of a non-significant rise in the number of incorrect responses made, introduction of a noise trial possibility into the 5CSRT task did not adversely affect performance. However removal of the noise challenge significantly affected performance. Accuracy rose following removal of the noise trials. The rise in accuracy was probably due to the removal of the elevated incorrect response rate seen during noise trials. However, accuracy did remain significantly higher than it was before noise challenge. These indices might suggest that the unexpected lack of the noise challenge has resulted in increased vigilance on the part of the mice leading to the increased accuracy and slower latencies. This suggests that the 6CSR task is more difficult than the 5CSRT task leading to their improved performance at the simpler task. However, we have previously observed that mice often perform well on their initial exposure to a modified task, whether that task is of increasing complexity or not.

The *ad. lib.* feeding challenge was only carried out in the mice. A brief restoration to *ad. lib.* feeding, (18 hour free-feeding period) was sufficient to significantly alter performance in the task in a strain independent manner. *Ad. lib.* feeding resulted in an increase in omissions and an increase in response latency that are taken as signs of performance impairment.

Conversely an improvement in accuracy was seen following *ad. lib.* feeding. The increase in omissions in particular suggests a lack of motivation that is consistent with a state of satiation on an appetitive task. Previously LH rats have been shown to respond slower on a operant feeding schedule following either constant food access or 15 min access immediately before testing (Eagle, Humby et al., 1999). In the 5CSRT task a similar satiation-related reduction in speed would invariably result in a combination of increased omissions and increased correct response latency. This is exactly what was seen here. The increase in accuracy seen in our study might suggest the mice were only responding when they were certain of being correct or when the distance was not too great.

Overall the LH rats were particularly sensitive to alterations in task timing. They were heavily impaired by increasing the task inter trial interval with a fourfold increase in omissions (4.4 ± 1.2 to 20.7 ± 2.2 between 2 sec and 8 sec ITI, mean ± SEM) and an eightfold increase in anticipations (7.3 ± 2.7 vs. 57.2 ± 2.7 between 2 and 8 sec ITI). When exposed to a noise trial the LH rats responded inappropriately at chance levels, implying they were willing to respond when a stimulus was not given. In comparison Stolerman et al, (Stolerman, Mirza et al., 2000), did not show an increase in omissions when raising their ITI from 5 sec to 20 sec. They did however show an increase in omissions when the ITI was decreased to 1 sec. In both manipulations they showed a decrease in accuracy.

7 Study 6 Delayed Non matching to Position task

7.1 Introduction

Following success in developing a mouse model of visual attention using the 9 hole box it was decided to develop an equivalent to the Skinner box task using this apparatus. Matching to location tasks have already been reported in mice using plus or T-mazes and purpose-built mouse Skinner boxes. It was intended to develop software to allow a version of the delayed non-matching task to be run in our large test chambers with mice. The approach was analogous to the classical Skinner box task used in rats except the subjects were to be trained to respond to light stimuli instead of responding at extendable levers.

Arachnid software was adapted from that previously written for rat operant boxes by Dr Marston. The 9 hole boxes previously described (Section 2.2 and Figure 2.1) were modified so that only three holes were uncovered, the central hole (Hole 3 of the mouse 5CSRTT set-up) and the holes to the far left and the right (Holes 1 and 5 of the mouse 5CSRTT set-up). This allowed maximum spread and thus maximum discrimination of the stimulus locations whilst still being within a proven operating field for the mice.

7.2 Initial time based strategy

Preliminary studies generated a direct analogue to the standard rat delayed non-match to position task (DNMTP task). These studies progressed from the habituation procedures outlined in Section 5.2.1, 5.2.2 and 6.2.3 directly into the full task that included a time dependent delay phase between the cue and choice phases. The details of the full task are given in Section 7.3.2 but a brief description follows. The full delayed non-matching to position task was composed of three phases: cue, delay and choice phases. In the cue phase either the left or right hole would be lit as the cue. A response at the cue location initiated the delay phase where the central response hole was lit and remained active until a predefined delay had expired. After the delay had ended the next response in the central location would initiate the choice phase. In the choice phase the central hole was inactivated and the left and right stimulus holes were activated. If the subject responded in the same location as the last cue presentation they failed the trial resulting in a time out similar to that used in the 5CSRT task. A response in the opposite location to the cue presentation resulted in a correct trial leading to reward.

However in these preliminary studies the majority of the mice developed strong location biases almost exclusively responding in either the left or the right hole in the choice phase. To counteract this tendency the mice underwent forced correct training. In this training the preferred response location was deactivated when it was inappropriate to respond there. This forced the subjects to get the trial correct as only the correct location was active. After ten consecutive trials using this regime the subjects were allowed a further ten trials with both correct and incorrect locations active. This regime was continued for approximately one week but failed to show any noticeable improvement in non-preferred responding. This contrasts with experience in training rats where strong response location biases can often be broken within two remedial sessions. Furthermore, observation of the mice showed few were making perseverative responses in the central response hole. This lack of appropriate responding generated chance performance at the longer delays as the subjects were observed to begin exploratory behaviour and appeared disinterested in the task. Following this initial failure it was decided to switch the time-dependent delay into a fixed ratio delay, thus requiring a certain number of central hole responses to initiate the choice phase. It was hoped this would give the subjects a more response-orientated task than the time-delayed variant. In this new paradigm the delay was randomly chosen from a fixed ratio array of 1, 2, 4, 8, or 16 responses. All holes were active but unlit (except the central hole which was lit) during the delay phase. If any response was made in an inappropriate location (any hole other than the central hole) before completion of the current fixed ratio the ratio counter was reset thus delaying the eventual completion of the FR and the trial. This was subsequently limited to two resets per trial to prevent a subject being stuck on a particular delay *ad. infinitum.* However even this alteration did not prevent strong biases developing and relatively low numbers of trials being completed. It was decided therefore to address the issue of developing strong perseverative responding earlier in the training.

7.3 Final task using FR training and food restriction only

7.3.1 Introduction

The ultimate aim of this section of work was to develop a task that used a series of randomly presented time delays to probe the short-term memory of a mouse. However the early work showed the mice had difficulty making the transition from tasks where a response results in an immediate change in the environment be it reward or failure to a randomly delayed task. The 5CSRT data showed the mice were susceptible to impairments in performance by unexpectedly increasing the ITI, even though the novel ITI remained consistent over an

entire session. At a 4 sec ITI performance was significantly impaired in the C57 group although not the 129P2 group, the impairment was even stronger at 8 sec ITI. However these temporal disruptions were being forced onto a task with well-defined timing characteristics so the performance deficit could not be purely attributed to the imposition of a delay, rather they represent the impact of the unexpected.

The progressive ratio studies described in Chapter 5 demonstrate that the C57 mice were capable of maintaining a repeating response pattern in order to obtain reward. By necessity the higher FR levels required the subjects to respond repeatedly over a longer period of time without a visible change in the local environment. Although this task used an incrementally escalating FR as opposed to a randomly generated FR it did show the mice were able to respond over longer periods of time. It was decided that integration of this type of ascending fixed ratio paradigm into the training protocol might have allowed the mice to perform the transition to a delayed non-matching task more readily.

7.3.2 Materials and training

A group of 16 male C57Bl/6J mice were used in this study. They were group housed and maintained on food restriction.

Stage 1 Habituation to reinforcer, as previously mentioned by giving milk whilst still on *ad. lib.* feeding (Section 5.2.1.).

Stage 2: Habituation to the test equipment, again the same as used with the 5CSRT task (Section 5.2.2.).

Stage 3: Simple response training. This marked a divergence from the 5CSRT task training by using a single hole. The programme used was the training programme described in Section 5.2.3. Initially, 20 consecutive trials under the target latency were required for FR increment. This was maintained for 6 sessions then the number of repeats was reduced to 5 consecutive trials. This allowed the subjects to receive 20 trials at FR1 before advancing to the more difficult FR2 during their preliminary 6 training sessions. To encourage responding at the higher ratios it was then necessary to reduce the number of repeats required to 5, even though this made the task easier. The reduction in required repeats meant that subjects were not so restricted by intermittent slowly-completed trials that would cause a resetting to the repeat counter. This allowed the subjects to progress to higher FR more readily.

Stage 4: Addition of a randomised cue phase. In order to develop responses to other lit holes without losing performance in the previous task this stage was designed. The FR phase noted above, now designated the delay phase or minimum retention interval was preceded by stimulus presentation at either the left or right holes as the cue. A single response at the lit hole resulted in inactivation of the cue hole and initiation of the delay phase described above. Retrieval of the eventual reward was followed by the ITI then another randomised cue phase. Advancement to the final task required attainment of an FR6 on this stage.

Stage 5 Final non-matching to place task. The task was divided into three phases: cue, delay and choice phases. The Cue phase was as in Stage 4 with the left or right stimulus being presented (i.e. beam activated and hole lit) in a randomised balanced manner. The cue hole remained active until a response was made in it, no contingency was attached to any other response. A response in the cue hole resulted in cue inactivation and immediate activation of the delay phase. In the delay phase the central response hole was activated and remained active until a predetermined delay had lapsed. The delay was randomly selected from an initial array of 1, 2, 3, 5 and 8 sec. delays. The central hole was inactivated following the first response in the central hole after the delay period had elapsed. Inactivation of the central hole began the choice phase. In the choice phase both the left and right stimulus holes were activated. A response in the location where the cue was provided was an Incorrect Response which resulted in an immediate Time Out (TO) period similar to that used in the 5CSRT task, namely immediate inactivation of all holes and activation of the house light for 5 sec. After the timeout period had elapsed the magazine was activated and a response at the magazine began the inter trial interval (ITI) prior to the next trial's cue phase. A response in the opposite hole to the cue location was a Correct Response resulting in reward. The reward protocol was the same as in the 5CSRT task, namely all holes are inactivated, the magazine activated and milk delivered to the lit magazine. Again a response in the magazine began the ITI prior to beginning the next cue phase. Later versions of the protocol included a 4 sec. reward duration added to the ITI on correct trials. Initially this was not deemed necessary unlike in the 5CSRT task because in this task the cue phase would not end until responded to. However, observations suggested the mice responded better to changes in the immediate environment. Thus a cue that came on during observation was apparently more salient than a cue that was already on. Hence they responded more readily to a cue that appeared whilst they were attending to the array and this required an increase in the period allowed for consuming reward. Although initially the programme ran for 20 minutes and used a random delay array of 1, 2, 3, 5, 8 sec this was finally extended to 40 minutes and used an extended

delay array of 1, 2, 4, 8, 16 sec. Data was analysed from the final version of the task only as the acquisition phase was compounded by multiple manipulations to the programme listed above. The primary index of performance used was accuracy; this was defined by:

Equation 7.1

$$Accuracy = \frac{Total\ Correct\ Responses}{Total\ Presented Trials}$$

There were ten possible trial groupings, left vs. right cued over five possible delays. With ten possible permutations of trials the number of trials at any particular trial permutation could be very low within a session. Therefore the data from two consecutive sessions was collapsed together and accuracy was recalculated from the total correct over the total presented and not just taking the mean accuracy over the two sessions. The frequency of responding in the central hole during the delay phase was measured by beam break rate. Beam break rate was calculated as total beam breaks within a delay grouping by session divided by the total trials presented with the delay grouping. This gave a mean beam break rate per subject per session for each of the five delay settings. These values were then averaged to give mean beam break rate by delay for paired sessions

7.3.3 Results

Performance was assessed by total accuracy independent of cue location. This allowed a larger sample size at each of the delays by combining both left and right cued trials. Two way Repeated Measures ANOVA showed a significant main effect of paired session ($F_{(14, 840)} = 2.08$, $p = 0.014$) and delay bin ($F_{(4,840)} = 17.28$, $p < 0.001$). A significant interaction between the two was also seen ($F_{(56,840)} = 1.79$, $p < 0.001$). *Post hoc* analysis showed that the performance on the first paired session was significantly lower than every other session at the shortest delay (1 sec) and for the later sessions at the next shortest delay (2 sec). There were no significant session effects on the higher delay trials (delay 4 – 16 sec). Overall performance was significantly better at the 1 sec delay than at any other delay (Figure 7.1).

Figure 7.1 Mean accuracy

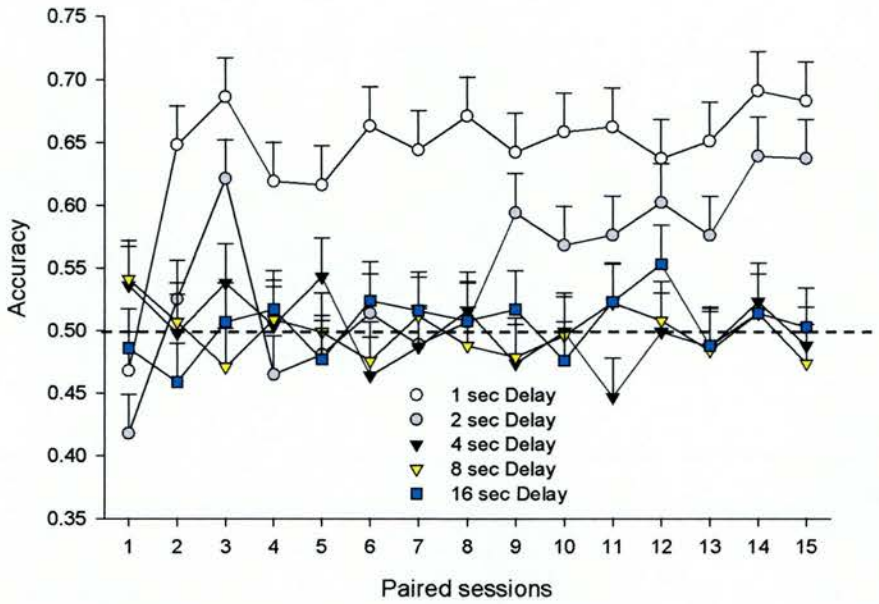


Figure 7.1 shows the mean accuracy of responding by delay independent of cue location. Data is plotted as group mean + SEM of 16 subjects from paired sessions. Dotted line indicates chance performance at 0.5.

The number of responses made (beam breaks) at the central hole during the delay period was counted to investigate whether the subjects were learning to increase their response rates at the longer delays over time. A significant effect of delay bin ($F_{(4,840)} = 1496.2$, $p < 0.001$) and session ($F_{(14,840)} = 7.7$, $p < 0.001$) was seen using logged data. A significant interaction was also seen ($F_{(56,840)} = 3.5$, $p < 0.001$). Overall, number of beam breaks made was significantly higher on paired session 1 than at any other time (Figure 7.2). Furthermore, beam break per trial rose significantly at each of the delay bins with highest number of beam breaks occurring on the longest delays. This effect is probably due to the time limitation to respond in at the lower delays.

Figure 7.2 Beam break

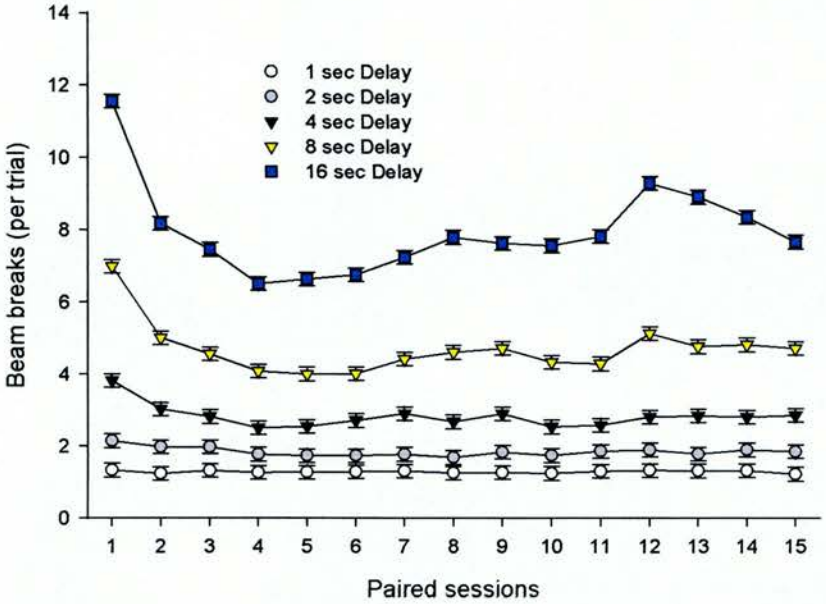


Figure 7.2 shows mean number of beam breaks made within a single trial binned by delay. Beam break numbers were calculated as total beam breaks within a delay by session / total trials by delay with the session. Data is plotted as group mean ± SEM of 16 subjects from paired sessions.

Figure 7.3 Total trials

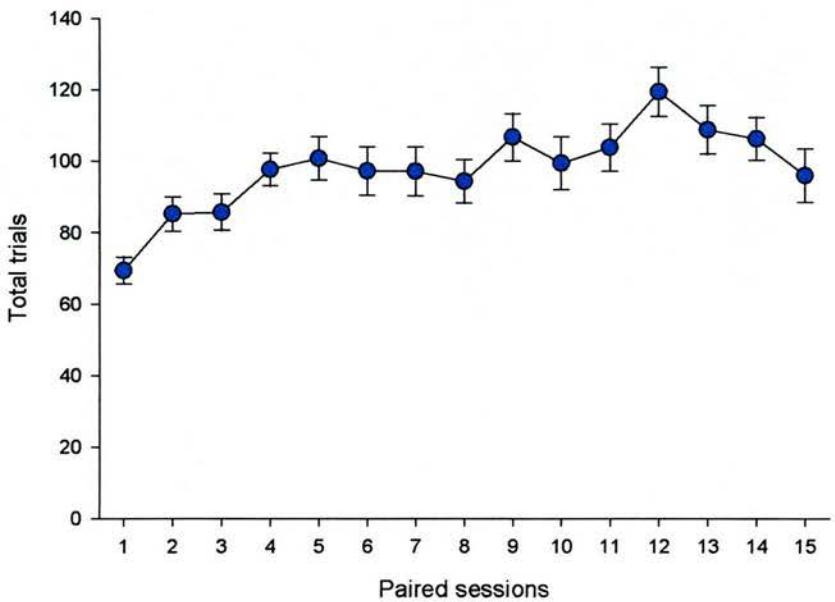


Figure 7.3 shows total trials (group mean ± SEM of 16 subjects) over paired sessions.

The maximum trial number in this task was the same as for the 5CSRT task, 120 trials. Due to the increased time taken to complete a single trial in this paradigm the session length was increased to 40 minutes rather than the 5CSRT tasks 25 minutes. However, no subject managed to complete 120 trials in this task within a single session. One-way Repeated Measures ANOVA of the total trials completed showed a significant effect of paired session on trials completed ($F_{(14,210)} = 6.0$, $p < 0.001$). Tukey's *post hoc* analysis showed this was almost exclusively due to the low trials completed on session 1 (Figure 7.3).

7.3.4 Discussion

It proved difficult to clearly demonstrate any evidence of working memory being used in this task. Trial numbers remained low throughout which was compounded by the ten possible trial permutations. Observations indicated that the mice commonly adopted a 'shuttling' behaviour wherein they would respond at hole 1, hole 3 then hole 5 in sequence on a hole 1-cued trial. If the delay was not over by the time they reached hole 5 they backtracked and eventually reached hole 1 again. After this sideways motion if the delay was still not over the subjects tended to occupy themselves with exploratory behaviour before returning to the response array again. The rat 9 hole box provides ample space to move around in and several areas that are possible to climb. It was common to observe mice climbing inside the chambers rather than attending to the response array. It is possible that with a more restrictive arena such inappropriate behaviours would be reduced. However, these behaviours are not commonly seen in the mouse 5CSRT task that uses the same test chamber implying a task-related cause.

Although both groups of subjects managed above chance performance of 50 % accuracy, it was only at the shortest delays (1 and 2 sec). Disappointingly the mice were unable to clearly demonstrate above chance performance at the 4 sec delay. At the 8 and 16 sec delays performance remained at chance levels throughout the 15 paired sessions. In comparison, the strain used here, C57Bl/6J, required 25+ days to reach acceptable performance in the 5CSRT task. Furthermore, in the 5CSRT task it was shown that C57 mice were slow to acquire a novel paradigm so this strain characteristic may have impeded progress in this task. However, the data presented here does not indicate any improvement in accuracy over the sessions especially in the longer delays implying that they had reached asymptotic performance in this variant of the task. In comparison, Lister Hooded rats have been shown

in this lab to be capable of much greater accuracy (~ 95 – 100 % accuracy at short delays) in the operant protocol this task was derived from.

7.4 Discussion

This chapter only used the C57 substrain due to the preponderance of prior studies using this strain for behavioural experiments. Although we have demonstrated the 129P2 strain were faster at acquiring the 5CSRT task there were initial difficulties in getting the 129P2 mice to accept the milk reward. This reluctance to sample sweet liquids has been previously reported by Lush (Lush, 1989) as a low preference for sweet substances observed in the related 129/Sv substrain. Failure of the initial studies here demonstrated the importance of appropriately shaping the subjects by inclusion of perseverative response training. We were able to demonstrate that the C57 substrain was capable of perseverative responding consistently reaching 8 to 10 responses in order to attain reward (Chapter 5). The rationale behind placing importance on developing perseverative responding to the central hole was to try to limit mediating strategies on the part of the subjects. If the subjects remain at the central hole they are limited to orienting their bodies to indicate the correct direction in the choice phase. The delay-dependent effect may be a true mnemonic limit with this substrain using the paradigm as designed. The same strain has been reported to perform non-matching choices at greater than 80 % accuracy on up to 10 second delays using a classical two lever operant box (Estape and Steckler, 2001;Estape and Steckler, 2002). In their paradigm the response levers were cued by stimulus lights above the levers which may have aided accuracy but this increases the differences between our exclusively light-cued task and their published task. This implies there was some problem inherent with our experimental design rather than an inability on the part of the mice. The short delays used rely purely on working memory or mediating strategies to accomplish the task. Unlike many working memory tasks our task uses few spatial cues, the darkened test chamber possesses few of the strong extra-maze cues seen in equivalent radial and T-maze non-matching tasks that may assist in accurate performance.

Furthermore the DNMTP task described here does not remove the mice from the test chamber during the delay phase (retention interval). In water maze tasks the subjects are invariably removed to a spare cage during the retention interval thereby making it impossible for then to make further responses within the test arena until the delay is complete. Similarly in T-maze and radial arm maze paradigms the subjects are at least restricted to the start location during the retention interval. In comparison, the mice in the 9 hole box are not

removed during the retention interval, so they are capable of making inappropriate responses throughout the interval. Although all responses are deliberately programmed to be non-contingent during the delay phase this cannot stop the subjects developing inappropriate strategies based on non-contingent responding thereby impeding learning. Even in the commonly-used operant Skinner box from which this task is based this potential confound is usually removed. This may help explain our failure in contrast to Estape and Steckler's relative success in this task using mouse skinner boxes. Matching and Non-matching to place tasks in the Skinner box usually include the withdrawal of the levers during the retention interval. This has the benefit of preventing the subject from making inappropriate responses during the retention interval. Thus, without the addition of a means to prevent inappropriate responding, by automated closure of the response holes or some other means, this variant on the DNMTP task may never provide a successful model of working memory in either the mouse or the rat. This argument is strengthened by the demonstration that C57 mice were capable of acquiring both an attentional and a working memory task using the same apparatus elsewhere (Durkin, Beaufort et al., 2000;Leblond, Beaufort et al., 2002). In this report behaviour was measured using a 5-arm maze that essentially replaced the response holes with covered wedge-shaped chambers. The arms could be individually lit to indicate rewarded location. In the visual attention task the subjects were rewarded by correctly responding at the lit chamber. In the delayed matching task the mice were given a forced correct sample (only one location open) followed by removal from the chamber for the entire duration of the retention interval. These mice were capable of performing a matching task. Interestingly these mice were initially trained on a "one strike out" protocol, i.e. so that the first wrong location selected in the choice phase finished the trial. Whilst on this protocol the mice were unable to perform at better than chance (20%). Their performance only improved after modifying the protocol to allow them to make errors in the choice phase. After several training sessions where all errors were non-contingent they were returned to the normal single mistake equals failure protocol and were able to maintain good performance. However this is akin to the forced correction protocol mentioned in Section 7.2 which was deemed to be ineffective.

8 Study 7 Circadian phenotyping of a novel mutant mouse

8.1 Introduction

Both vasoactive intestinal peptide (VIP) and pituitary adenlyate cyclase-activating polypeptide (PACAP) have been shown to have an impact on circadian rhythmicity *in vitro* and *in vivo*. VIP has been shown to phase advance rat SCN rhythmic firing when applied in early subjective night *in vitro* (Reed, Meyer-Spasche et al., 2001). *In vivo* VIP was shown to cause time-dependent phase shifts when injected directly into the hamster SCN (Piggins, Antle et al., 1995). PACAP when applied to SCN tissue *in vitro* could phase advance the timing of rhythmic firing in a time-dependent manner (Hannibal, Ding et al., 1997). These phase advances were only seen during the SCN day. *In vivo*, PACAP administration has been shown to cause large transient phase advances in hamster running wheel activity. Again these effects were predominantly seen following daytime administration (Piggins, Marchant et al., 2001). Both of these ligands can bind to the $VPAC_2$ receptor (Harmar, Arimura et al., 1998) which is heavily expressed in the mammalian SCN (Ibata, Okamura et al., 1999). Although there are now some antagonists for the $VPAC_2$ receptor there have been no published reports of the effects of these on circadian behaviour as yet. It was therefore decided to investigate the effects of the generation of a VPAC2 receptor -/- (knock-out) mouse line to investigate any circadian outcome of the absence of this receptor. The behavioural outcomes of this null mutant line are described here.

8.2 Materials and Methods:

$VPAC_2R$ KO and corresponding control wild type mice were generated as described by Harmar (Harmar, Marston et al., 2002). The $VPAC_2R$ KO mice were derived from a genetically-modified 129/Ola-derived ES cell line implanted into C57Bl/6J females. The resultant chimerae were then bred with C57Bl/6J mice to generate the F1 generation. The F1 generation were then bred together to create the F2 generation including some KO mice. These KO mice were then bred with C57Bl/6J mice to create heterozygous mice who were then repeatedly bred against further C57Bl/6J generations to create a KO line with a C57Bl/6J and 129P2/Ola background.

The experimental set-up is described in Section 2.3. using the larger Tecniplast wheel (240 mm diameter, 80 mm wide) with ten null mutant and fourteen wt mice. The behavioural

indices were similar to Chapter 4, overall activity, effects of phase advance and phase delay on activity onset and free-running rhythm in dim red light were measured as described previously. Additionally the effects of exposing the subjects to brief (2 hour) bursts of dim red light during the 'inactive' half of their LD cycle were examined. For this part the group sizes were increased to 15 subjects per group. Response to the novel 'Dark bursts' were analysed in terms of proportion of subjects active within the dark burst and lag in onset of activity following dark burst commencement.

8.3 Results

The data for the activity counts was not normally distributed due to the separation between diurnal and nocturnal activity. Log transforms failing to restore normality. Non-parametric ANOVA analysis using Sigmastat was limited to one way ANOVA on Ranks, therefore it was decided to Rank transform the data and use a parametric analysis. Using this approach the data set passed normality, this was only required for the activity comparison. Mean locomotor activity was calculated as the mean of a ten day period in either the 12 hr light or dark phase (Figure 8.3). RM ANOVA showed a significant effect of phase ($F_{(1,22)} = 171.48$, $p < 0.001$) but no effect of strain ($F_{(1,22)} = 2.69$, $p = 0.115$ n.s.) using ranked data. Using the untransformed data the phase effect remained. *Post hoc* analysis showed a significant increase in activity in the dark phase in comparison with the light phase with the wt group being twofold more active than the KO mice in the nocturnal phase (mean ± SEM of 12163.6 ± 2026.3 vs. 5122.9 ± 970.2 counts per 12 hour period). Conversely, the KO mice were more active in the diurnal part of the cycle (mean ± SEM of 270.5 ± 126.6 vs. 85.1 ± 17.8 counts per 12 hour period). To compare the effect of an absence of light on daily activity, total daily activity was calculated over 10 days in constant darkness. This was compared with total daily activity over 10 days under the standard LD cycle immediately preceding the constant dark period. Analysis showed the level of activity was dependent on the regular presence of light, ($F_{(1,22)} = 5.04$, $p = 0.035$) using two way RM ANOVA and was weakly dependent on strain, ($F_{(1,22)} = 4.346$, $p = 0.049$). An interaction between the two main effects was also seen ($F_{(1,22)} = 23.1$, $p < 0.001$). *Post hoc* analysis showed that the mean total activity was highest under a 12 hr light 12 hr dark regime but this was only significant within the WT group (Figure 8.4).

Figure 8.1 Sample $VPAC_2R$ KO actogram

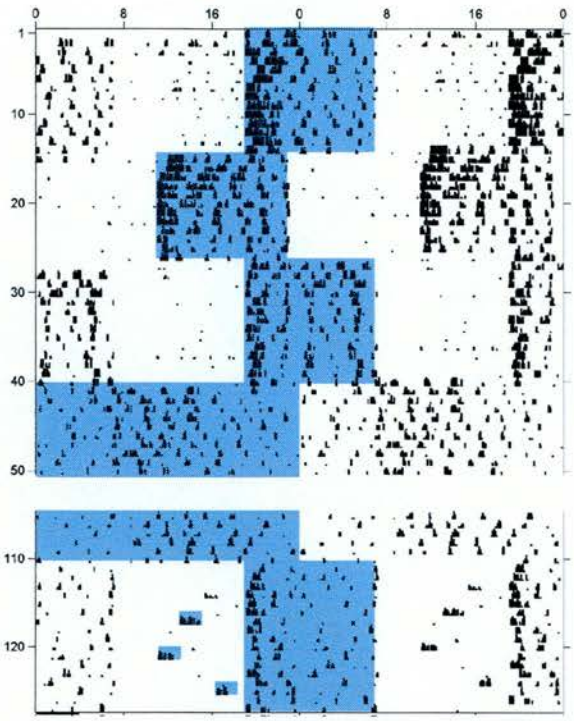
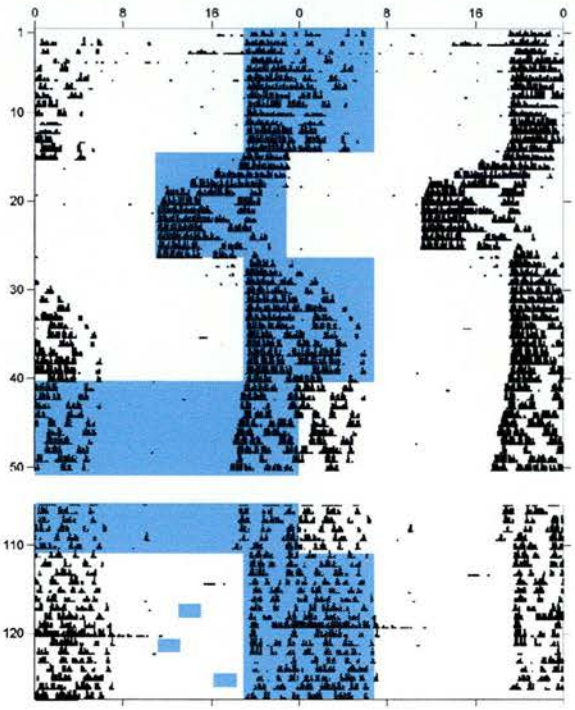


Figure 8.2 Sample Wild type actogram



Sample actograms from the $VPAC_2R$ KO (Figure 8.1) and wt (Figure 8.2) mice are given. The actograms are laid out as described in Chapter 4. The actograms generated from all the subjects in this study are given in Appendix B.

Figure 8.3 Diurnal vs. Nocturnal Activity

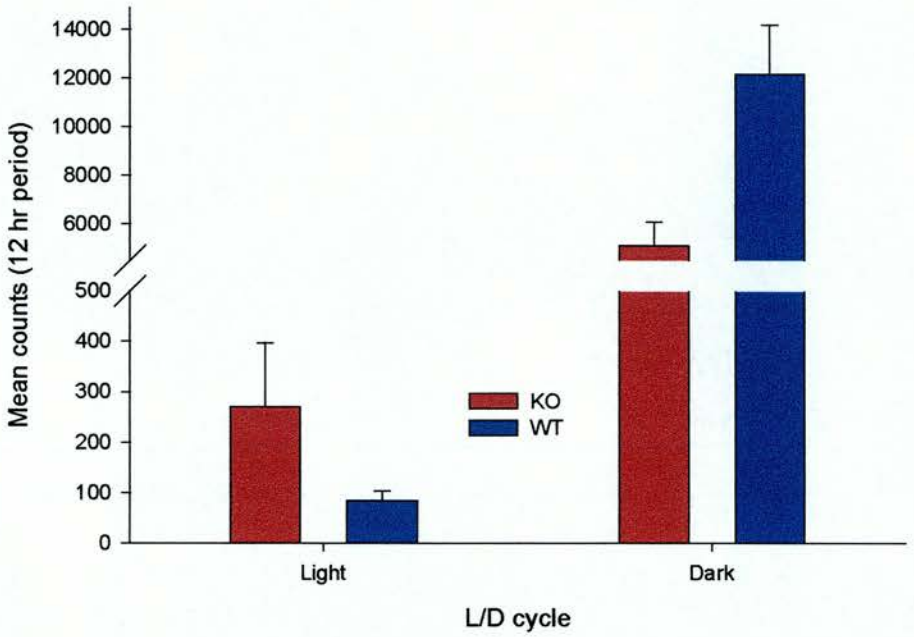


Figure 8.3 shows mean ± SEM counts over the 12 hour period in either light or darkness over ten consecutive days following entrainment.

Figure 8.4 L/D vs. DR Activity

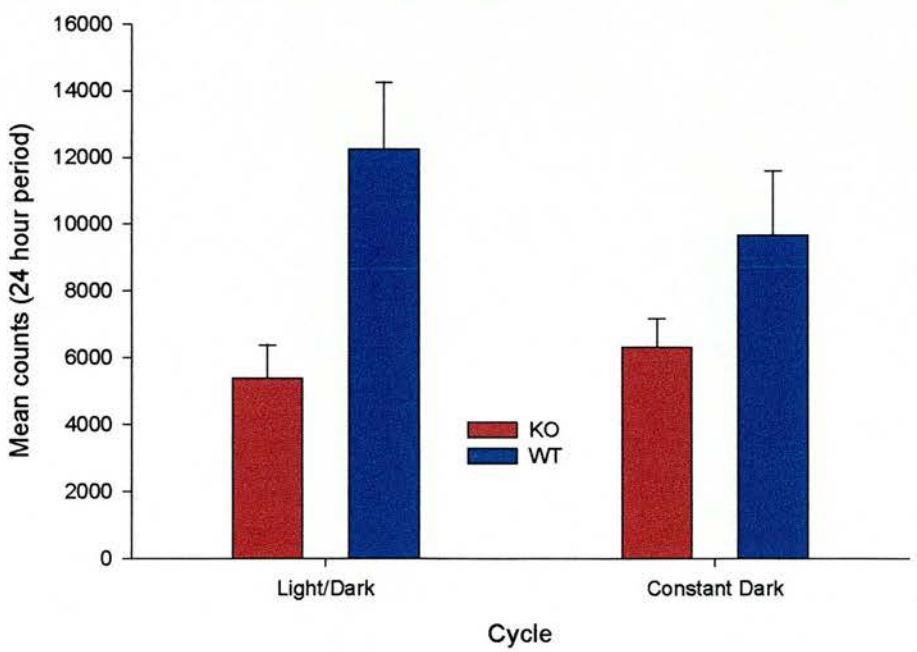


Figure 8.4 shows mean ± SEM activity over the 24 hour period under either a 12:12 L/D cycle or constant darkness over ten consecutive days.

Figure 8.5 Effects on re-entrainment

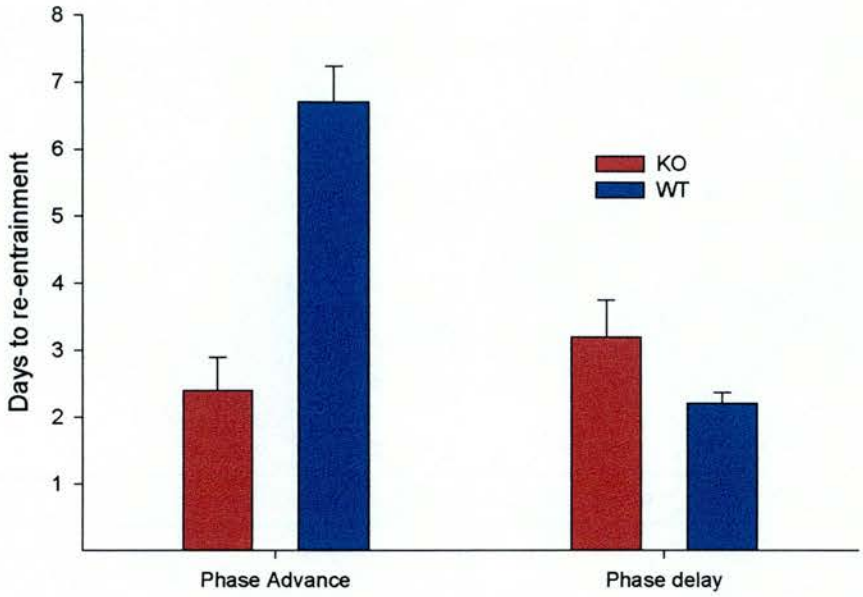


Figure 8.5 shows mean ± SEM days to re-entrain following either an 8 hour phase advance or 8 hour phase delay in the onset of darkness.

The shift to a novel LD cycle caused both genotypes to re-entrain over time. Two way RM ANOVA of the days taken to re-entrain showed this was strain- ($F_{(1,22)} = 11.17$, $p = 0.003$) and shift-dependent ($F_{(1,22)} = 21.14$, $p < 0.001$). A significant interaction was also seen ($F_{(1,22)} = 43.38$, $p < 0.001$). *Post hoc* testing showed that the KO group shifted significantly faster than the wt group during the phase advance but not during the phase delay (Figure 8.5). Analysis of the endogenous free-running rhythm in these mice was also carried out. However, free-running rhythms were only reliably obtained for the wt mice (tau, $\tau = 23.9 \pm 0.04$ hours from $n = 14$ mice).

Following the rapid response to the unexpected shift in darkness onset and the disruption to activity in the absence of white light it was also decided to investigate the effects of brief bursts of darkness during an otherwise normal L/D cycle.

The lag in onset of activity following each dark burst was calculated by strain. Two way ANOVA showed no effect of the timing of the dark bursts but did show a significant effect of strain ($F_{(1,57)} = 17.4$, $p < 0.001$). *Post hoc* analysis showed that the KO mice became active significantly sooner after dark burst onset than the wt mice (0.28 ± 0.06 hours vs. 0.75 ± 0.09 hours). Furthermore, 96 % of the KO mice were active during the three dark burst exposures

compared to only 43 % of the wt mice. In this case 'active' was defined as 'showed any activity during the 2 hour dark burst.

8.4 Discussion

Free-running rhythms were only reliably obtained for the wt mice. This was due to the fact that under constant dim red light conditions (DR) the pattern of activity in the KO mice was grossly disrupted. Rather than maintain a cohesive activity pattern in DR with identifiable periods of activity and inactivity the KO mice became active throughout the 24 period. The algorithm used to identify time of activity onset and hence tau in the Clocklab software used the best fit for a model of 6 hours inactivity (persistent activity below 20 % of all non –zero counts) followed by 6 hours of activity (persistent activity above 20 % of all non-zero counts). With no distinct period of inactivity in the KO mice under DR conditions it was very difficult to generate a reliable activity onset time and hence the inability to define a meaningful tau value. However, the same data period (first 10 days under DR) was subjected to a Fast Fourier Transform (FFT) that does not rely on bouts of activity and this showed a 24 hour rhythmicity in the KO as well as wt mice (Harmar, Marston, Shen, Spratt, West, Sheward, Morrison, Dorin, Piggins, Reubi, Kelly, Maywood, and Hastings, 2002). This mutant presents the first evidence of an intracellular signalling knockout that causes complete disruption to both the behavioural and molecular rhythms in mice. Previously only mutations to the molecular clock itself have caused such complete breakdown. Knock out of either both Cryptochrome (*Cry1* and *Cry2*) or both mouse Period (*mPer1* and *mPer2*) genes have been shown to cause arrhythmia in constant conditions (van der Horst, Muijtjens, Kobayashi, Takano, Kanno, Takao, de Wit, Verkerk, Eker, van Leenen, Buijs, Bootsma, Hoeijmakers, and Yasui, 1999;Zheng, Albrecht, Kaasik, Sage, Lu, Vaishnav, Li, Sun, Eichele, Bradley, and Lee, 2001).

9 Study 8 Single choice visual discrimination task

9.1 Introduction

The previous chapters have described significant differences between unrelated inbred mouse strains. Chapter 8 describes some innate behavioural outcomes of a gene manipulation. It has been shown that the 9 hole box coupled to appropriately designed experiments can be a sensitive tool for measuring inter-strain differences. Following the description of substantial effects of the $VPAC_2R$ knockout on classical circadian behaviour it was decided to investigate whether or not the genetic manipulation had altered sensitivity to visual stimuli in a non-circadian manner.

Although a measure of the sensitivity to visual stimuli has already been described for the 5CSRT task (Section 6.3.2) it was recognised that the approach was not very sensitive. Furthermore, the time taken to train subjects to respond accurately in the full five choice task was unnecessary when all that was required was to train subjects to respond to a single location. By reducing the task to the detection of the presence or absence of a single stimulus the results become amenable to signal detection theory. This approach can prove very powerful in analysing the sensitivity, in this case to a visual stimulus, and response bias simultaneously (Marston, Sahgal et al., 1993;Marston, 1996). To this end the 9 hole box was modified to leave only a single central hole open and active. In order to calculate the SDT parameters appropriately the probability of a response or lack of response to a signal or background noise needed to be derivable. These probabilities are described in Table 9.1. From these parameters indices of sensitivity (SI) and response bias (RI) can be calculated. Animals were initially shaped to perform a simple response task before being advanced to the SDT task. Finally the protocol was used to examine the effects of manipulating stimulus intensity on SI and RI.

9.2 Materials and methods

Groups of $VPAC_2R$ knockout and wild type littermates (n = 8 animals per group) were used in this study though one wild type mouse died prior to entering the final phase of training. Subjects were given free access to water and food-restricted as previously described. Subjects were initially group-housed.

Training in the single choice discrimination task was as follows.

9.2.1 Habituation phase

Habituation to the reinforcer (3 days under *ad. lib.* feeding) then habituation to the test equipment (2 days) was carried out according to Section 5.2.1 and 5.2.2.

9.2.2 Simple response phase

The protocol from Section 6.2.3 was modified so that the single exposed response hole was activated and lit. A response here elicited reward. This was maintained for one week. The programme was then modified to include a stimulus duration (SD) of 10 sec, a failure to respond within the SD resulting in the immediate activation of a 4 sec time out (TO) period. As with previous protocols the time out phase was marked by the illumination of the house light and inactivation of all other lights. Following time out the magazine was reactivated and a response here began the ITI period prior to the next stimulus presentation. During this phase the session length was increased to 20 min and the stimulus duration reduced in a stepwise fashion to 8, 4, 2 and finally 1 sec. The programme was subsequently refined to allow individual stimulus durations to the multiple test chambers within a single session. If the number of time outs was greater than the number of rewarded trials the subject was placed on the next stimulus duration in ascending order until number of rewarded trials was at least double the number of time outs.

9.2.3 Signal detection task

This phase required the insertion of a secondary stimulus probability. The programme listed above was modified so that for each trial there was an equal probability of the bright stimulus being presented (signal present) or a blank stimulus being presented (signal absent). This was similar to the noise challenge listed in Section 6.3.4. A response during the signal stimulus duration was categorised as a Hit, a failure to respond to the signal was a Miss. A response during a blank stimulus (signal absent) was categorised as a False Alarm and a lack of response to the blank stimulus was a Correct Rejection. A Hit response was always rewarded, and a Miss or a False Alarm response was always punished with a time out and the house light coming on. A correct rejection was neither rewarded nor punished with the house light and time out delay. These contingencies are defined in Table 9.1 below. The ITI

was increased to 5 sec, and time out decreased to 3 sec. Both bright and blank stimuli retained the same stimulus duration of 2 sec for all subjects. Although some subjects were capable of accurately responding at a 1 sec SD, others did not appear capable of this so the slightly higher setting was used to include all subjects. A 1 sec limited hold was now included as well (see Section 6.2.3). All subjects were retained on this protocol for a minimum of 12 sessions.

Table 9.1

	Response	No response
Signal Present (Bright stimulus)	Hit	Miss
Signal Absent (Blank stimulus)	False Alarm	Correct Rejection

9.2.4 Stimulus intensity challenge

For the final phase the equipment was reconfigured so that each test chamber could have two possible stimulus intensities, governed by the resistance across part of the circuitry. Expression of each intensity setting was via activation of a specified input line to the test chamber. The programme used in Section 9.2.3 was altered so that the two stimulus possibilities were linked to the separate input lines thus allowing two possible stimulus intensities to be presented within a single session. Stimulus intensity was calibrated daily for each test chamber. The light meter (Model RS180-7133, RS Components Ltd., UK) detector was placed directly over the central hole and all other sources of visible light were extinguished. Stimulus intensities were set using the following logarithmic scale; Max, 300, 100, 30, 10, 3, 1, 0.5, and 0 lux. Maximum stimulus intensity was different for each test chamber and but it was higher than 300 lux in all chambers. The Arachnid programme used in this phase is reproduced in Appendix C as an example of the software used throughout the appetitive testing presented here.

The study ran in two cycles, in cycle 1 stimulus A (signal) remained at normal levels (>300 lux) and stimulus B (noise) cycled from 0 to 300 lux and back again using a single daily session at each intensity. In the second cycle stimulus B (noise) remained constantly at 0 lux and stimulus A (signal) cycled from 300 to 0 lux and returned to 300 lux. Response contingencies were described using Table 9.1 above with stimulus A as the signal. Comparison between the two cycles was possible through comparing the difference between signal and noise levels (Difference = Signal strength – Noise Strength).

Performance was calculated through analysis of the ratios of p(Hit) and p(FA) respectively using the number of trials within each category for the following equations:

Equation 9.1 and 9.2

$$p(Hit) = h = \frac{Hit}{Hit + Miss}$$

$$p(FA) = f = \frac{False\ Alarm}{False\ Alarm + Correct\ Rejection}$$

p(Hit) and p(FA) are calculated separately for each individual by day. According to Frey (Frey and Colliver, 1973) measurements of sensitivity (a form of response accuracy) and response bias can then be described by using the following equations.

Equation 9.3 and 9.4

$$SI = \frac{(h - f)}{2(h + f) - (h + f)^2}$$

$$RI = ABS\frac{(h + f - 1)}{(1 - (h - f)^2}$$

9.3 Results

There were no significant effects of the mutation measured using this single choice paradigm under either constant signal or constant noise conditions.

In the first iteration, using a constant maximum strength signal and variable noise, there was a significant effect of session on both sensitivity and response bias, ($F_{(12,156)} = 79.71$, $p < 0.001$) for sensitivity index and ($F_{(12,156)} = 3.89$, $p < 0.001$) for response bias. Tukey's *post hoc* analysis confirmed that SI fell as the noise level increased and the difference between signal and noise was reduced (Figure 9.1). SI reached a significant nadir at the maximum noise level and minimum signal to noise difference on session 7. As the noise level was reduced performance recovered once again. Conversely response bias increased at the maximum noise levels before dropping as the difference between signal and noise increased once again.

Figure 9.1 Effects of increasing noise on a constant high signal strength test

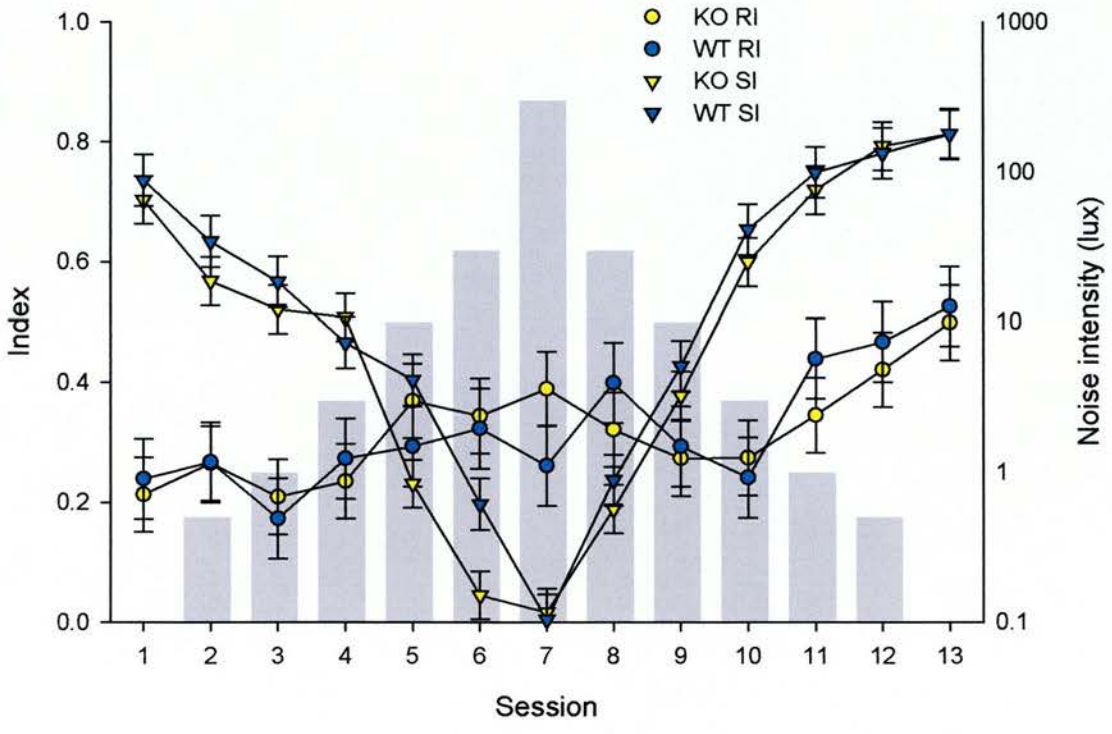


Figure 9.1 shows the effect of increasing noise on a constant high strength signal trial. The histogram plots intensity of noise presented (lux) against the logged right hand axis. Left hand axis plots the mean ± SEM sensitivity (triangle) and bias (circle) index values derived from the wild type (blue symbols, n = 7) and $VPAC_2R$ KO (yellow symbols, n = 8).

The second iteration used a constant low level of noise and reducing signal strength protocol. Again there were significant effects of session on both SI and RI, ($F_{(14,182)} = 57.42$, $p < 0.001$) and ($F_{(14,182)} = 3.11$, $p < 0.001$) for SI and RI respectively. Tukey's *post hoc* analysis

showed that SI dropped significantly as the signal strength was reduced with performance on session 8 (difference = 0 with signal and noise at 0 lux) significantly worse than on any other day (Figure 9.2). Performance also recovered as signal strength and hence difference increased once more. In this iteration there was a more pronounced increase in RI at the minimum difference point (session 8) but this was only significantly different from a limited number of the other sessions.

Figure 9.2 Effect of decreasing signal strength on a constant low strength noise test

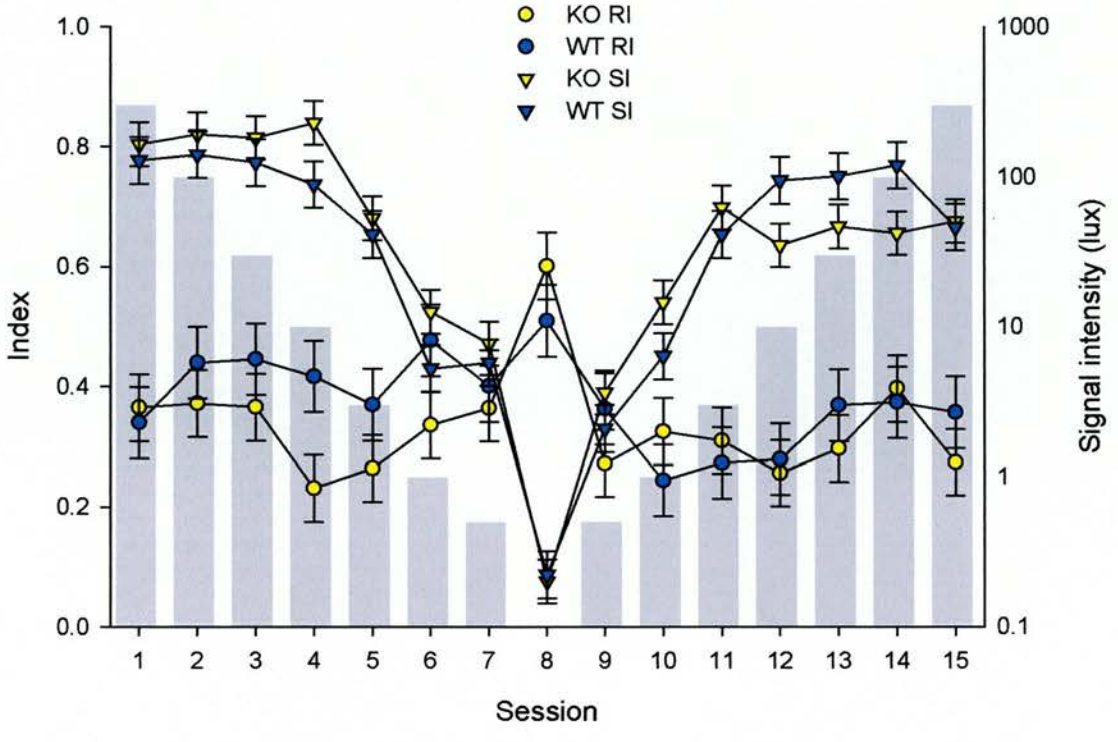


Figure 9.2 shows the effect of decreasing signal on a constant low noise trial. The histogram plots intensity of signal presented (lux) against the logged right hand axis. Left hand axis plots the mean ± SEM sensitivity (triangle) and bias (circle) index values derived from the wild type (blue symbols, n = 7) and $VPAC_2R$ KO (yellow symbols, n = 8).

9.4 Discussion

This task failed to show any dissociation between the $VPAC_2R$ KO and WT mice in detecting brief pulses of light. In both cycles accuracy of responding, measured by SI, decreased as the difference between signal and noise strength decreased to 0. Unfortunately the first cycle used a maximal signal strength (> 300 lux) and the noise strength only reached

300 lux, hence even at the maximum noise there was a slight difference between the two presentations. The nadir was flattened in the first cycle, possibly due to the difficulty of discriminating a bright signal from an almost equally bright noise. The nadir of the SI in the second cycle was much steeper, due to the potentially easier task of detecting a very dim signal 0.5 lux in comparison to a non-existent noise (0 lux) as in sessions 7 and 9. Interestingly, there appears to be little impairment on performance of the task in the second half of each cycle. It was anticipated that at the mid point of the cycle where signal and noise strengths were equal the apparent randomness of reward vs. punishment for responding would result in interference in future performance, this was not seen.

10 Discussion

10.1 Inbred mouse circadian differences

The results show there were significant differences between the inbred mouse strains examined under a 12/12 hr L/D cycle. The C57Bl/6 mice were approximately twice as active as the 129P2/Ola mice during the Dark period in the wheels. This corresponds with reported differences between these two strains for horizontal locomotor activity (Royle, Collins et al., 1999). Additional studies comparing the C57Bl/6J strain with other 129 substrains 129/SvJ (Miner, 1997;Homanics, Quinlan et al., 1999) and 129/SvEvTac (Crabbe, Wahlsten, and Dudek, 1999) give a similar pattern. The disparity between the two strains was even greater during the Light period – this may suggest the C57Bl/6J mice weren't under such strict light-induced locomotor inhibition as the 129P2/Ola strain.

It has been suggested that the 129P2/Ola mice possess poorer co-ordination or balance as evidenced by approximately 50 % lower latency to "falling off" on the rotarod test when compared to C57Bl/6J mice (Royle et al, 1999). It could be reasoned that poorer co-ordination would impede their ability to run in the wheels, especially at speed. However, motor co-ordination and balance measured by duration balanced on a thin elevated bar; a variation on the wire test detailed in the SHIRPA protocol (Rogers, Fisher et al., 1997); indicated that 129P2/Ola co-ordination improved substantially (twofold greater duration on bar) over the course of just two days (Montkowski, Poettig et al., 1997). This would suggest that any initial co-ordination difficulties encountered regarding the wheels could be in part overcome with practise.

It has been previously noted that free access to wheels can modulate circadian activity in mice (Edgar et al 1991) and attempts have been made to use wheels as positive reinforcers in learning tasks (reviewed by Kopp et al 1997). If wheel running is in itself positively reinforcing it is possible that the higher wheel-associated activity in the C57 strain may impair their active state during Light periods leading to the higher levels of light activity seen. Conversely, the more inactive 129P2/Ola strain would receive less positive reinforcement during the Dark period due to lower and more sporadic activity so would be more able to rapidly respond to the presence of light hence their lower Light period activity. We could reasonably expect that light activity resulting from positive reinforcement during the Dark period would be predominantly located at the beginning of the Light period. This is

not evident from inspection of the actograms; most Light period activity seems to be associated with random external noise cues during normal working hours.

Analysis of the strains' responses to acute shifts in onset of Light and Dark periods indicate that the mechanism of shift (either by a short or long day shift) is not significant. Under both conditions the 129P2/Ola mice, on average, adjusted to the new time of 'lights outs' (start of Dark period) within 35 hours of the start of the modified Light period. This equates to most subjects beginning onset of activity on the onset of the first Dark period following Light period perturbation. In the case of the short day shift this resulted in an immediate and sustained activity pattern upon 'lights out' even though it was 6 hours earlier than previously encountered. Under the long day shift the response required a repression of activity presumably due to extension of the 'lights on' period. With the other two strains (C57Bl/6 and CBA/Ca) the response was more gradual, resulting in greater periods of inactivity during darkness (short day shift) and greater activity during light periods (long day shift) until the onset of activity had reset to the new L/D cycle. This implies a greater reliance on the part of the 129P2/Ola strain on photic stimuli for entrainment. The slower resetting by the C57Bl/6J mice indicates that photic stimuli alone are unable to effect extensive alteration in activity very rapidly.

Investigation of the strain responses to an absence of photic stimuli similarly illustrated surprising disparity between the strains. Commonly subjects exhibiting a stable activity rhythm will maintain that rhythm in constant darkness, abet at a now modified frequency or circadian period. This pattern of behaviour is known as free running. The C57Bl/6J mice exhibited strong free running rhythms with a circadian period of 24 hours in constant darkness (DR) which wasn't significantly different from the activity seen in 12/12 hr L/D. In contrast, whilst the 129P2/Ola mice maintained a strong 24 hour rhythm in L/D that pattern became dispersed in DR, with the duration of the active period (alpha) extending beyond that seen in the other strains of mice. Restoration of the standard 12/12 L/D cycle caused immediate re-entrainment within the 129P2/Ola mice (data not shown). This would also strongly imply a pre-requisite for photic stimuli for maintenance of circadian activity by 129P2/Ola mice. This apparent photic stimuli requirement by the 129P2/Ola mice correlates well with the strong influence exerted by the modified light periods whilst phase-shifting the 12/12 hr L/D cycle.

C57Bl/6J mice are known to have abnormal melatonin release (the mammalian hallmark of subjective night). Unlike certain other strains of mouse they do not apparently have a clear nocturnal peak of melatonin (Kopp et al 1998). The reason for this seems unclear at present and to date I have located no information regarding melatonin release in 129P2/Ola mice. However, whilst circadian rhythmicity can continue unabated in pinealectomised rats, melatonin has potent entrainment effects. Administration of melatonin can entrain rats to new activity onsets (Redman, Armstrong et al., 1983) and can accelerate re-entrainment to L/D cycle phase shifts when administered at the time of new Dark onset (comment in Kopp et al 1998). Melatonin is known to act on, amongst others, the SCN. Neuronal firing within the SCN is circadian, peaking during daylight hours and at its lowest during darkness (Borjigin, Li et al., 1999). Melatonin appears to act on $GABA_a$ receptors (Wan, Man et al., 1999) which form a principal mediator of SCN activity resulting in inhibition of SCN neuronal firing (Wagner, Castel et al., 1997). Thus, the lack of a large melatonin peak in the C57Bl/6J mice may play a role in the extended time taken to adjust to the phase shifts in the L/D cycle used here.

It is generally agreed that 129 substrains of mice are difficult subjects for behavioural phenotyping. As well as poor performance in many behavioural paradigms (Crawley, Belknap, Collins, Crabbe, Frankel, Henderson, Hitzemann, Maxson, Miner, Silva, Wehner, Wynshaw-Boris, and Paylor, 1997) their lack of genetic homology between substrains and with other strains makes assessment of the impact of background genetics difficult to say the least. Therefore considerable effort has gone into deriving ES cell lines from other, experimentally more useful, mouse strains (Crawley and Paylor, 1997). Some success has already been reported in creating C57Bl/6 and BALB/c derived ES cell lines (Lemckert, Sedgwick et al., 1997). Advances are being made in the fields of reproductive biology that allow greater success rates with implantation of previously recalcitrant ES cell lines and blastocysts (Lemckert et al 1997) and mouse strains (Brook and Gardner, 1997). Unfortunately these advances have generated their own caveats, currently C57 derived ES cell are injected into BALB/c blastocysts creating another cross-strain interpretation problem (Lemckert 1997). Nevertheless, in the future, successfully injecting a C57-derived ES cell into a C57 blastocyst would considerably reduce the number of polymorphic loci creating genetic variability within litters since C57 substrains have relatively few polymorphic loci. Therefore recent developments have gone some way to providing not only the much sought-after single inbred background but also the genetically similar littermate control.

The theoretical ideal in murine molecular genetics currently is the generation of a "pure-bred genetic background". This is most easily conceptualised where the ES cell line is derived from the eventual recipient substrain and that the substrain has as few polymorphic loci as possible. However even this approach, whilst it could limit genetic variability within the litters, still leaves the problem of the intrinsic deficits of the mouse strain chosen.

10.2 Importance of activity phase

Traditionally animals are housed under 12 hour light and dark cycles and most experimentation is carried out during the light phase. Mice are generally nocturnal animals, our work has demonstrated an increase in locomotor activity upon onset of dark or inactive phase currently thought to be related to intrinsic foraging behaviour. Work by Valentinuzzi (Valentinuzzi, Buxton et al., 2000) showed that in an open field task C57 mice demonstrated the anticipated biphasic locomotor response over a standard 12/12 LD cycle. The mice were more active when tested during the dark phase than during the light phase independent of when in the particular phase they were tested. However, when the subjects were subjected to a constant dim green light and tested early in their active and inactive phases according to wheel-running behaviour this biphasic response was totally abolished.

This biphasic activity pattern is also seen with certain gene expressions, generally in the suprachiasmatic nucleus but also elsewhere in the body (Lopez-Molina, Conquet et al., 1997). Nelson illustrated the potential dangers of phenotyping only during the inactive phase with the example of neuronal nitric oxide synthase (nNOS) KO mice. Standard circadian behaviour revealed no significant differences between the two genotypes (Kriegsfeld, Demas et al., 1999) with both groups being able to entrain to phase advances and phase delays suggesting nNOS does not play an important role in circadian behaviour. Furthermore, when the nNOS KO mice were measured during the inactive phase they were equivalent to wt mice for both motor co-ordination and balance. However, when measured in the active phase, the nNOS KO mice were significantly impaired in contrast to wt mice (Kriegsfeld, Eliasson et al., 1999). This impairment was through a lack of improvement in the KO mice when tested in the inactive phase. Thus, where genes have biphasic expression, the timing of any behavioural analysis may be critical to determining any effects of a mutation. In order to assess whether a wt gene is phasically expressed there is a strong argument for analysis of gene expression over a 24 hour period under normal light: dark cycles before even generating a mutation.

10.3 5CSRT comments

The successful development of the mouse 5CSRT task both here and elsewhere opens exciting new areas for studying attentional deficits in neurodegeneration in particular. The large body of literature on serial response testing in various strains of rats give a useful starting point for examining mouse attentional performance. The robust genetic malleability of mice grants the experimenter the possibility of temporal dissociation of attentional deficits throughout development of neurodegenerative disease models. These are envisaged to be similar to the age-related working memory deficits reported in the water maze for Alzheimer's Disease models (Chen, Chen et al., 2000).

In some respects the mice appear to behave comparably to previous rat data. The age-dependent impairments in performance previously reported in rats (Jones, Barnes et al., 1995;Muir, Everitt, and Robbins, 1996;Muir, Fischer et al., 1999) have also been seen in mice (unpublished data). The data shown here indicates the serious impact disruption of the task timing can have on subjects used to a constant time frame. Temporal rather than cue-based strategies appear to be far more important to the rat than the mouse as the mice were reasonably unaffected by mild changes in task timing such as increasing ITI and introduction of a noise trial. However this did show some strain dissociation with the 129P2 mice being less affected by imposing a 4 sec ITI than the C57 group. In contrast, rat performance was significantly disrupted by imposing a longer ITI and especially by the introduction of a noise trial. The effects of increasing the ITI has been previously shown in a visual timing task (Broersen and Uylings, 1999). Broersen showed that Lister-Hooded rats trained to respond to a bright stimulus at a single location made significantly more premature responses when on a long (20 sec) ITI trial as compared to a short (10 sec) ITI trial. Interestingly there was no increase in omissions on longer ITI trials in these rats suggesting a lack of disengagement from the task. This further suggests the rats were still motivated to do the task. In contrast we have previously seen mouse performance tends to fall through a sharp increase in omissions at increasingly difficult tasks, which suggests a disengagement from attending which may be due to a lack of motivation. This tendency of mice to disengage from tasks may explain why they appear to respond favourably to stimuli changes timed to occur when they are looking at the stimulus array. An already active stimulus light does not appear to be as salient as a stimulus that changes states under observation.

The noise distractor trial used in this work suggests some interesting differences between how the mice and the rats performed the task. In the noise distractor challenge, the subjects

were required to respond to the lit stimulus as usual when it appeared. However, in one sixth of the trials no signal would appear, in these cases the subjects were rewarded for restraining from responding anyway. The mice showed excellent performance in this task, with greater than 80 % of the noise trials rewarded without any loss in performance of the standard task. In contrast the rats had difficulty performing the noise trial sessions. In the absence of a stimulus they responded anyway giving a chance level of performance on noise trials. This behavioural disinhibition is akin to the anticipatory responding seen in the other challenges. Impulsive responding in rats has been previously suggested to be associated to serotonergic pathways within the prefrontal cortex. Systemic administration of a 5-HT1A receptor agonist has been reported to increase impulsive responding in Long-Evans rats in the 5CSRT task (Carli and Samanin, 2000). This effect was reversed by application of a 5-HT1A receptor antagonist. However this manipulation also led to increased omissions and latency to respond which suggests the agonists effects are not purely through behavioural disinhibition. Reducing the stimulus intensity did not alter the agonist impairments showing that the serotonergic effects are not visually-mediated. Similar increases in omissions have been reported with a variety of other serotonergic agonists and 5HT depletion (Carli and Samanin, 1992;Harrison, Everitt et al., 1997). Dalley et al has also reported an increase in 5-HT efflux within the prefrontal cortex of Lister Hooded rats during impulsive responding (Dalley, Theobald et al., 2002). In contrast, the evidence for serotonergic effects in mice are less clear and are predominantly focussed on aggression-related impulsivity (Brunner and Hen, 1997). Some strains of mice have been shown to exhibit slow disinhibition of inappropriate nosepoking in an appetitively rewarded task (Logue, Swartz et al., 1998). In this task they were trained to nosepoke during but not before an auditory cue to receive reward. Nosepoking out with the tone enforced a delay before rewarded responding was possible. Certain strains persisted in inappropriate responding such as BALB/c whereas others adapted to appropriate responding quickly such as C57Bl/6. This implies we may have obtained far greater impulsive responding from the mice had we used a different strain.

The 5CSRT task described here has recently been adapted to utilise temporally as well as spatially random stimuli. Instead of a constant ITI, a randomly generated ITI from within a pre-defined range was used. The random presentation of stimuli would increase the sustained attentional load of the task, such that a subject would have to attend for far longer to perform accurately. The version described in this thesis involves a strong rhythmic component that integration of random stimulus presentations should help alleviate thus creating a more appropriate task. The data presented here suggests that random stimulus presentations might

be detrimental to performance, however other work in the laboratory suggests this is a learning effect. Mice trained using a random ITI learn to perform the 5CSRT task very accurately (Jared Young, unpublished data) suggesting the strategies used in our constant ITI task utilise some inappropriate rhythmic element. As yet we have not investigated the performance of rats trained on a variable ITI protocol.

10.4 Mouse strain recommendations

As discussed earlier mice provide a very useful tool for genetic modelling of human neurodegenerative disease amongst many other things. The extensive literature on rat and primate behavioural work provide a rich source of experience to develop comparable models in. We have added here to the vast body of literature that shows the phenotype of a mouse strain can have profound effects on the resultant behaviour. This is not a new issue, rat strain effects have been reported throughout the literature as well and most laboratories tend to favour specific strains dependent of performance or local availability. However, genetic manipulation brings an added layer of complexity to the issue. As has already been shown, the mouse strain being used in a task can have considerable impact on any mutations expressed on it and will greatly influence attempts at phenotyping in an effort to examine the mutations' functions. The debate concerning which mouse strains are most appropriate to use still continues. Some suggest that the best solution to homogenise data across experiments is to designate a single strain to which all genetic modifications are applied and assessed (Silva, Simpson, Takahashi, Lipp, Nakanishi, Wehner, Giese, Tully, Abel, Chapman, and et.al., 1997). Others such as Crawley (Crawley, Belknap, Collins, Crabbe, Frankel, Henderson, Hitzemann, Maxson, Miner, Silva, Wehner, Wynshaw-Boris, and Paylor, 1997) suggest that the intrinsic abilities of the various inbred mouse strains must be taken into account. They suggest that certain strains which already show 'aptitude' for certain behavioural tests e.g., C57BL/6 mice in learning related tasks, would make better carriers of targeted mutations hypothesised to affect those specific tasks. Furthermore, they recommend that where the effect of the mutation is uncertain that the strain chosen should have both capacity for improvement and decline in functioning. Sadly, this debate remains academic as long as we lack the technology to use the mouse substrain that we would ideally use under any given circumstance.

A single agreed strain onto which all genetic mutation would be placed seems to be the most practical way forwards in the long term. This strategy suffers from the fact that unless a carrier is created that has no endogenous deficits then there will always be a small subset of

mutations for which the carrier is inappropriate. Furthermore, unfortunate selection of the carrier strain may result in high levels of lethality following gene expression as seen with the EGFR mutation in 129/Sv mice (Sibilia 1995). By limiting oneself to a single carrier strain one would potentially lose the information gained by mutations interacting with several different carrier strains. However, once a particular strain is demonstrated to support mutations of a particular family of proteins without excessive lethality then clearly comparison of results across research groups would benefit from everyone using the same carrier strain. Utilising separate carrier strains for different paradigms, is better adopted for early exploration of novel mutations. The rationale of adopting specific carrier strains with well-known capability to perform in certain tests may allow easier identification of subtle mutation-dependent deficits or improvements. However, this approach may suffer should a mutation be analysed in a spectrum of paradigms when one carrier strain isn't appropriate for all tests. At this point the researchers would return to the current problem of whether to express their mutation in several strains or just accept poor performance from a single carrier strain in specific paradigms.

10.5 Conclusions

In conclusion we can see that many factors can have significant impact on measuring phenotypic perturbations following genetic manipulation. The behaviourist needs to know the methods used in creating the transgenic animal they are supplied with and the shortcomings of each method before drawing conclusions. In all cases the background mouse strains involved should be analysed to give some indication of whether one particular strain is particularly weak in the anticipated protocol. Ideally the mutation itself should be expressed on a single inbred background strain and where ES cells are used they should be also from the same strain where possible. Especially where this is not possible the mutation should be backcrossed onto an inbred mouse strain or F1 hybrid line for multiple generations but even then we must be aware that not all alleles from one parental strain will be bred out.

To comprehensively assess the effects of a protein multiple mutant lines would ideally be created. Mutants with the gene knocked out can indicate the effect of gene absence. On their own these mutant line have problems separating true gene absence from compensation effects. The inducible rescue mutant as well can demonstrate the acute effects of restoring gene function to a KO mutant. In these mutants, the issue of whether the gene products will have normal wt function upon restoration clouds interpretation. Finally the generation of an inducible KO mutant would allow normal development so avoiding the issues surrounding

compensation before altering gene expression. In all these cases, the accurate analysis of mutant phenotypes would be substantially aided by targeted gene alteration. The use of a relevant gene promotor could offer restricted gene expression both temporally and spatially allowing much finer discrimination of gene function.

We have attempted to establish a range of tasks that can test many aspects of cognition in mice. In most cases we were successful, developing a task of sustained attention that was amenable to both rat and mouse testing and have validated it in comparison to previously published rat data. We have successfully establishing a circadian protocol and used it to demonstrate a significant circadian mutant mouse. We have also validated our use of food restriction as a means of motivation. The DNMTP task was not particularly successful but other groups have reported tasks that successfully work in mice, and that address working memory. It is hoped that these tasks will be useful in testing genetic models of human disease and perturbation. The generation of appropriate models of human disease will require a broad range of tasks, both cognitive and physiological, that are sensitive to a wide range of deficits as exhibited by human patients.

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Appendices

Appendix A: Inbred Mouse Strain Actograms (i) – (iv).

The inbred mouse strain actograms represent the activity patterns of each individual subject in Chapter 4 over the 76 day period of the experiment. The y-axis plots consecutive days and the x-axis indicates time of day over a two day period beginning and ending at midnight (0 hrs). Most actograms are scaled to a maximum of 55 counts per bin (using 6 minute bins) to allow comparison between actograms. One 129P2 and three CBA/Ca actograms were too weak to be scaled to this level so were scaled individually. The weak 129P2 actogram (second row, left panel) was scaled to a maximum of 38 counts per bin. The weak CBA/Ca traces shown at second row (left panel) and third row (both panels) were scaled to 2.3, 3.9 and 0.8 counts per bin. The shaded areas indicate time of white room light off. All subjects were constantly exposed to dim red light.

Appendix B: $VPAC_2R$ KO and WT actograms (v) – (xii).

Parts 1a and 1b

These actograms represent the activity patterns of each individual subject (n = 10 for $VPAC_2R$ KO and n = 14 for wild type controls) in Chapter 8 over the initial 60 day period of the study. As with Appendix A, the y-axis plots consecutive days and the x-axis indicates time of day over a two day period beginning and ending at midnight (0 hrs). All actograms are scaled to a maximum of 80 counts per bin (using 6 minute bins) to allow comparison between actograms. The shaded areas indicate time of white room light off. All subjects were constantly exposed to dim red light.

Parts 2a and 2b

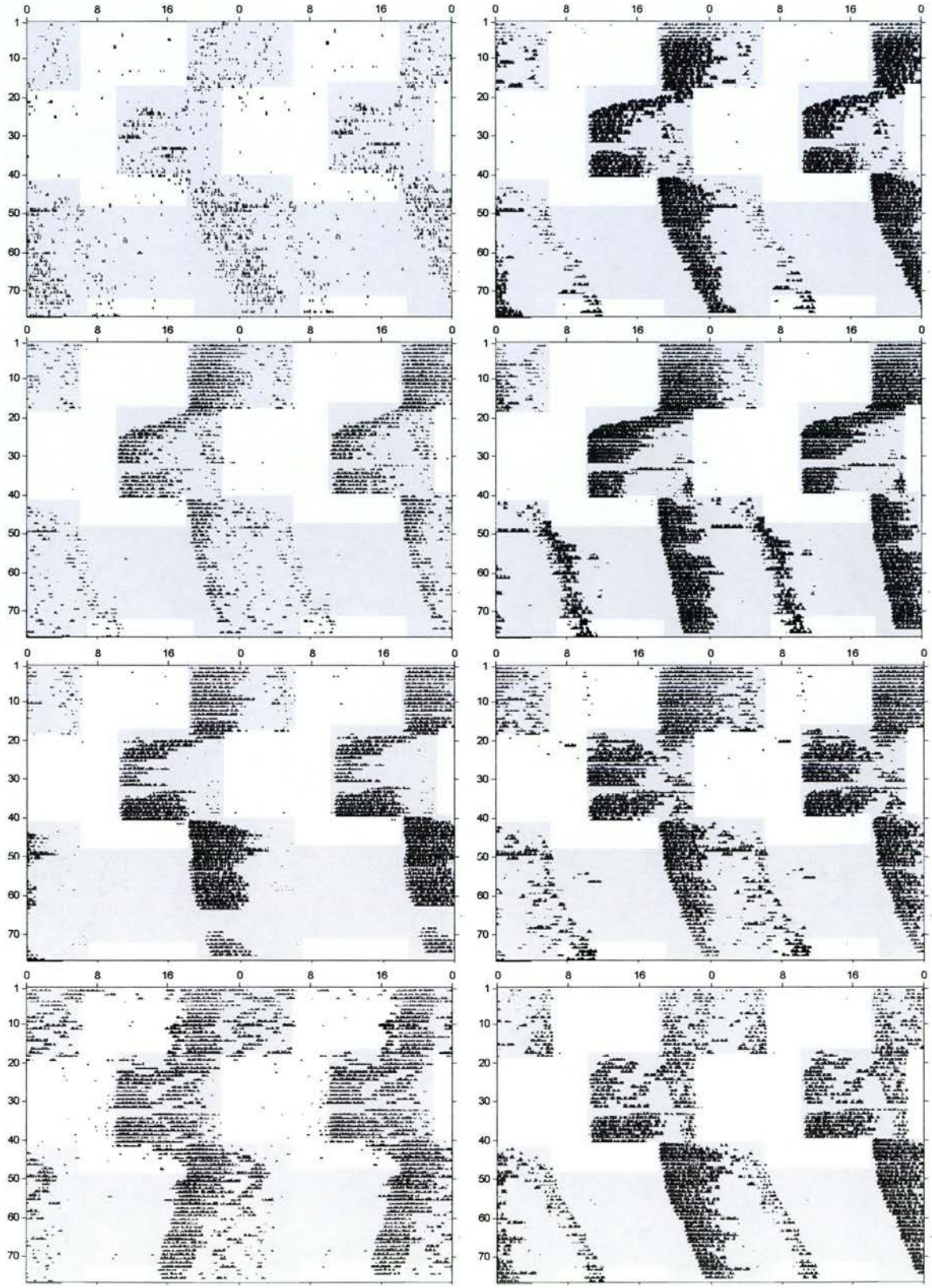
These actograms represent the activity patterns of each individual subject (n = 15 per genotype) in Chapter 8 over the final 25 day period of the study during which the dark pulse challenge happened. All actograms are scaled to a maximum of 80 counts per bin (using 6 minute bins) to allow comparison between actograms. The shaded areas indicate time of white room light off. All subjects were constantly exposed to dim red light.

Appendix C: Sample Arachnid programme (xiii) – (xvii).

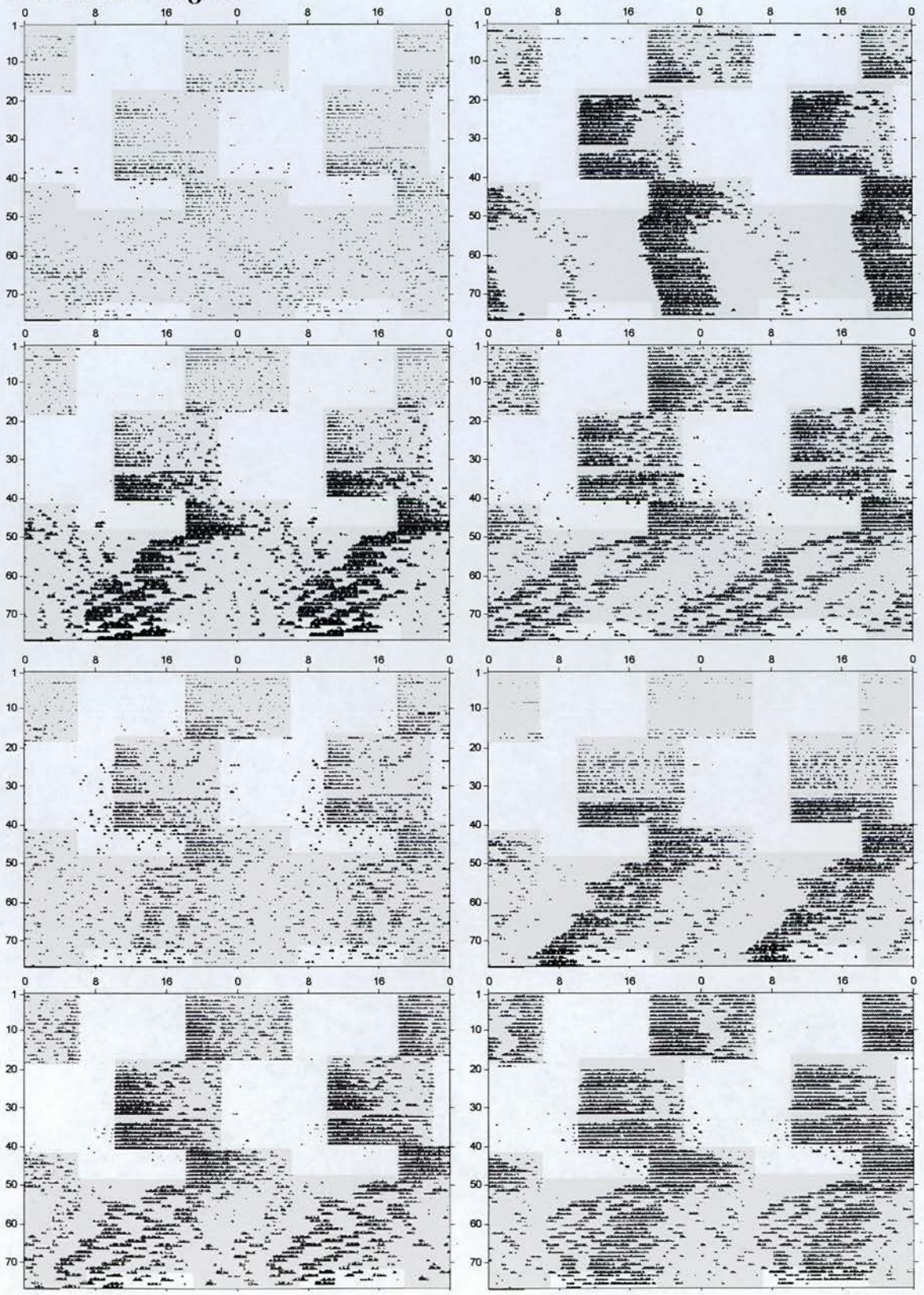
Sample computer programme as used in Chapter 9 for signal discrimination study. Programme was heavily modified by Mr C. Spratt from 5CSR programme written by Dr H.M.Marston.

Appendix A

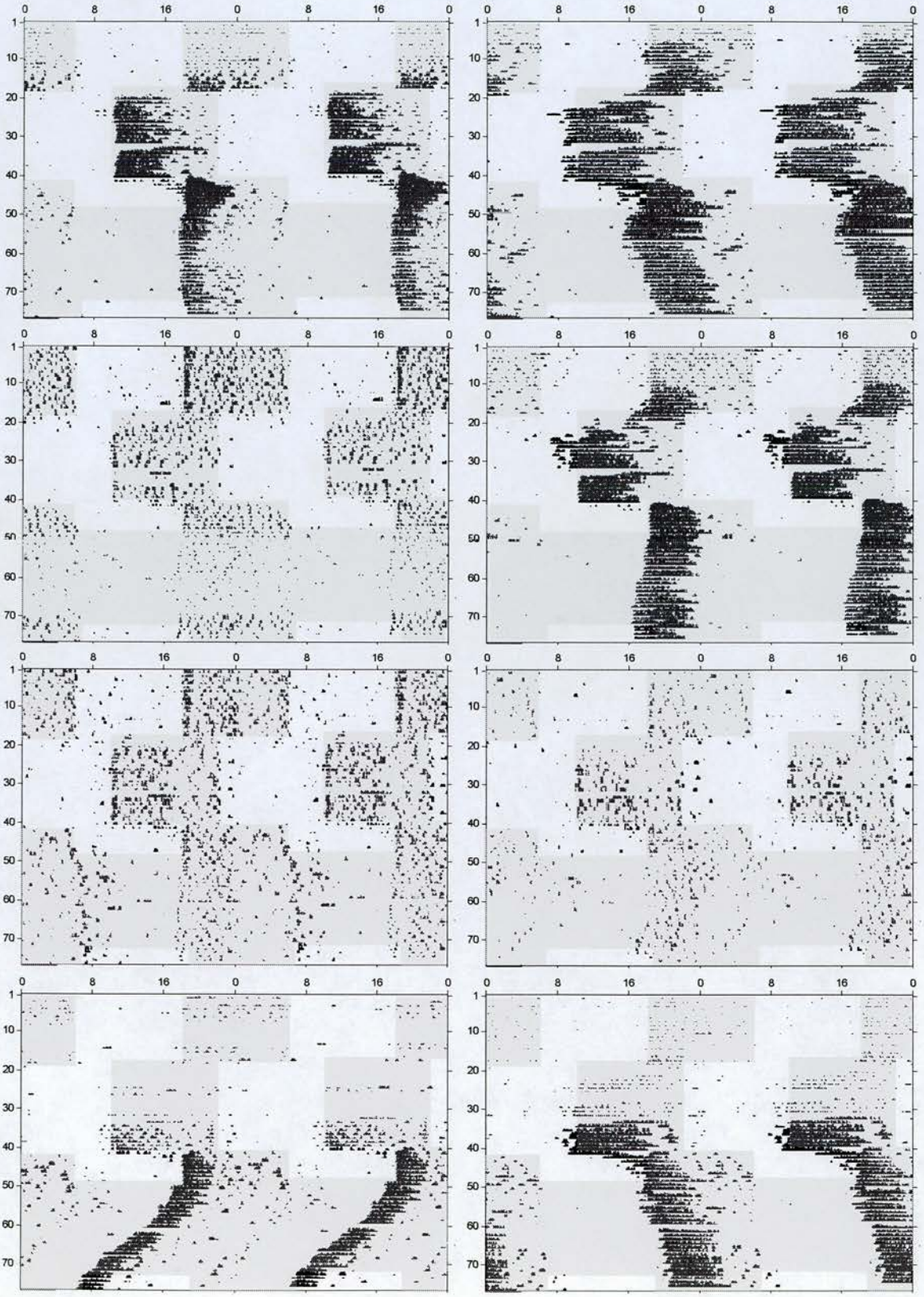
C57Bl/6J actograms



129P2/Ola Actograms

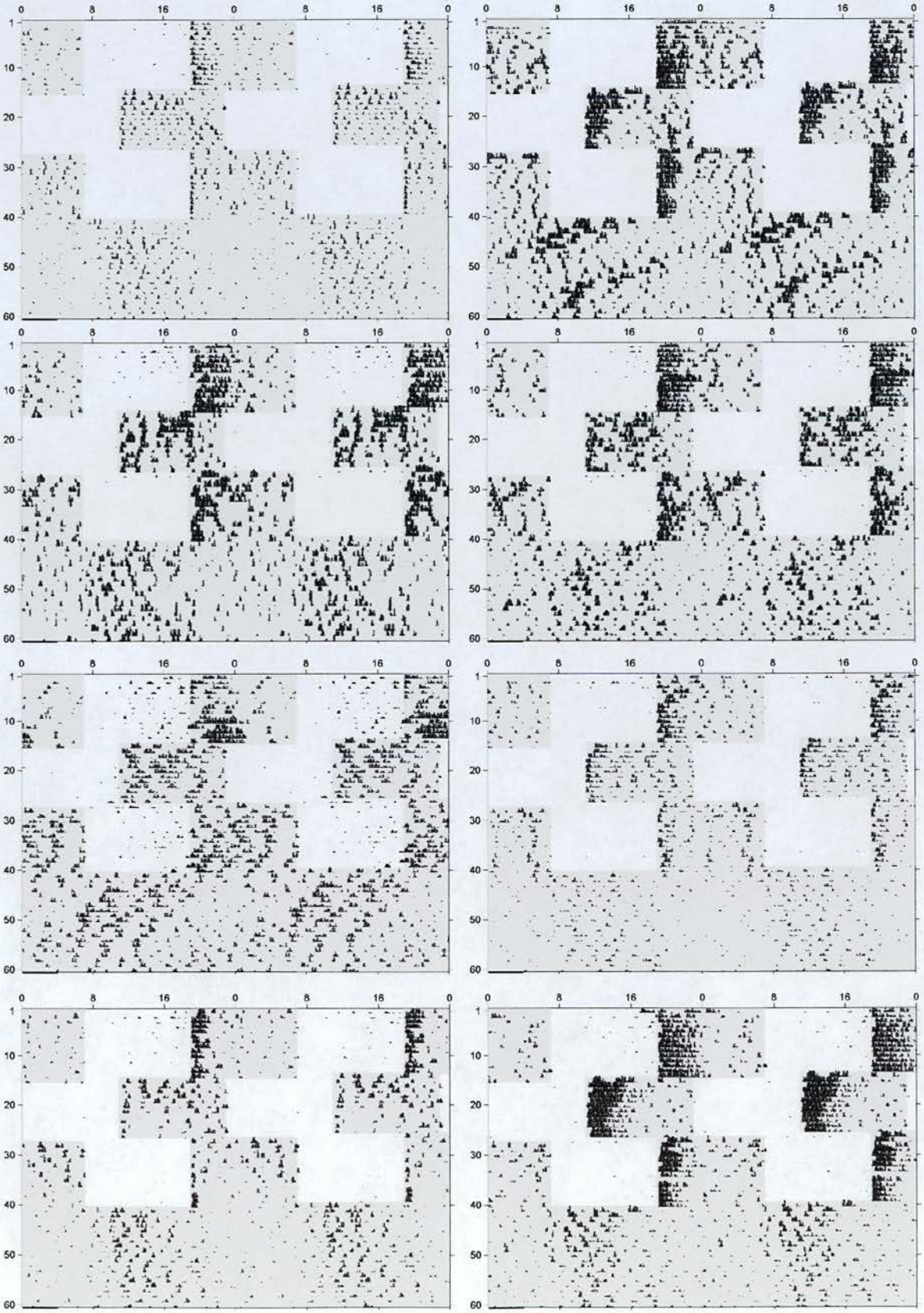


CBA/Ca actograms

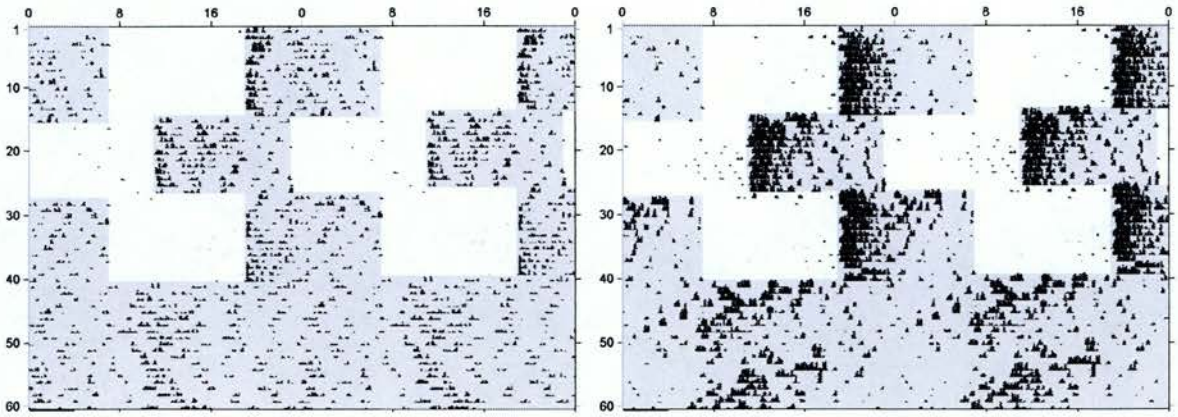


Appendix B

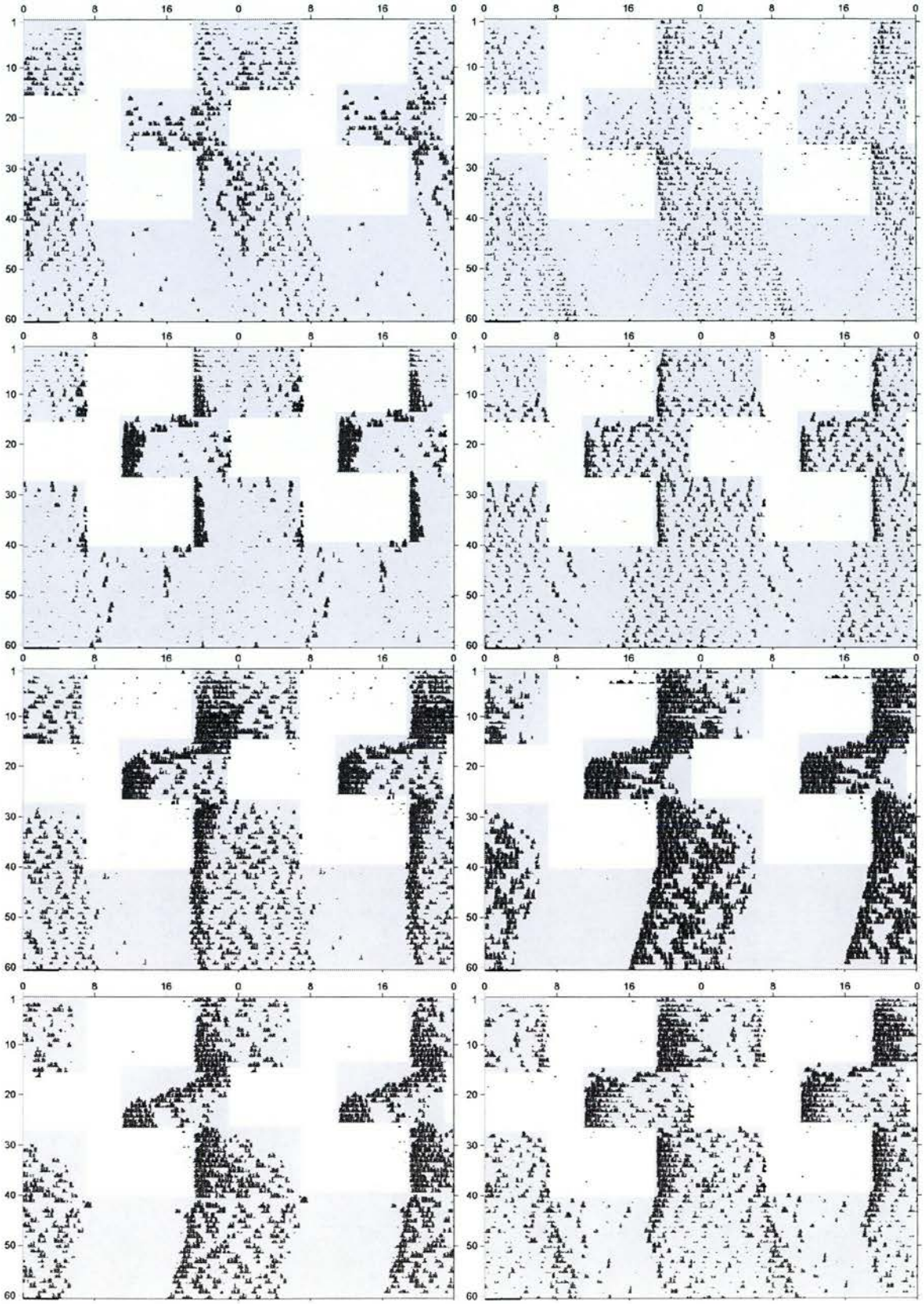
KO actograms Part 1a



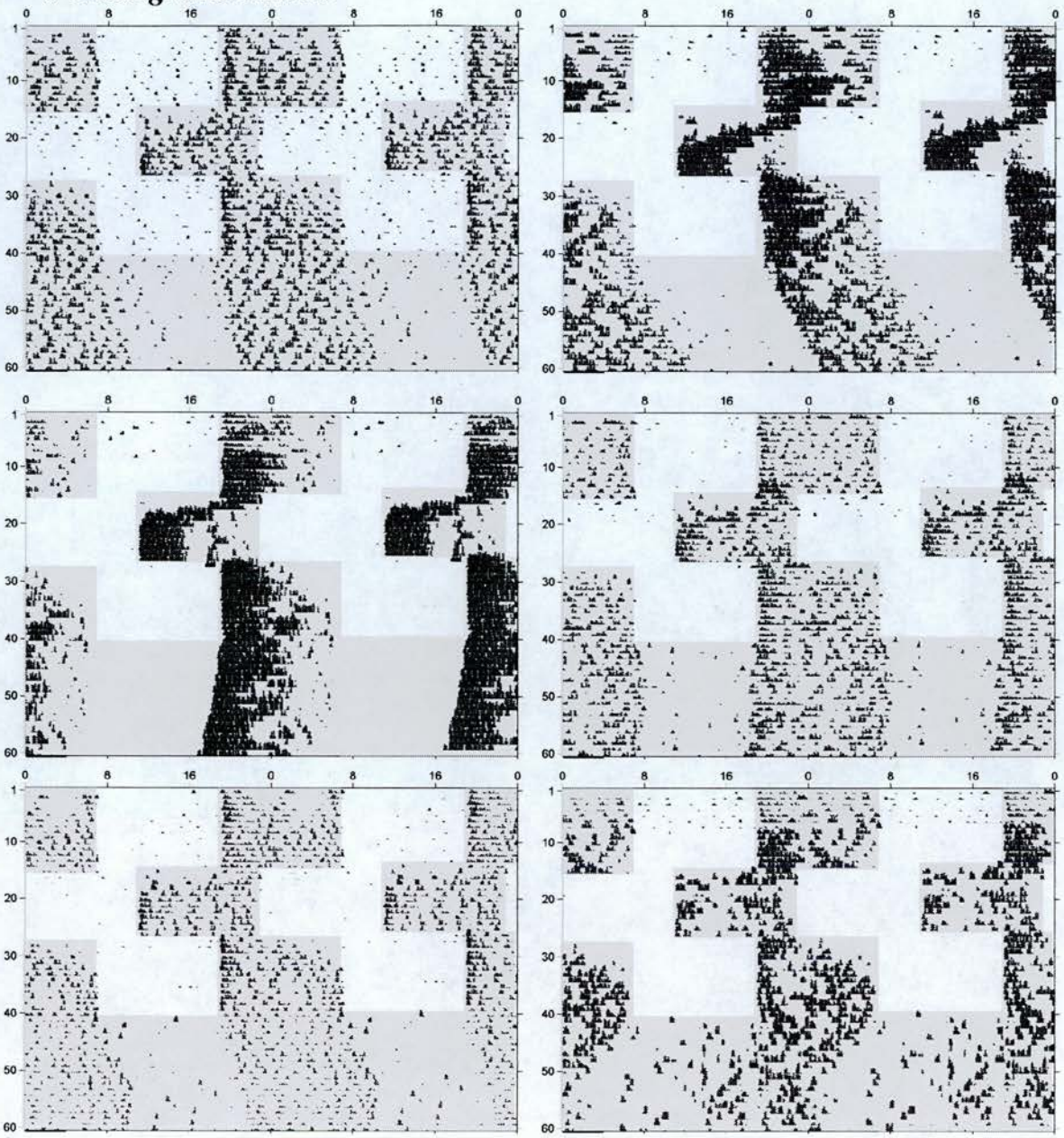
KO actograms Part 1b



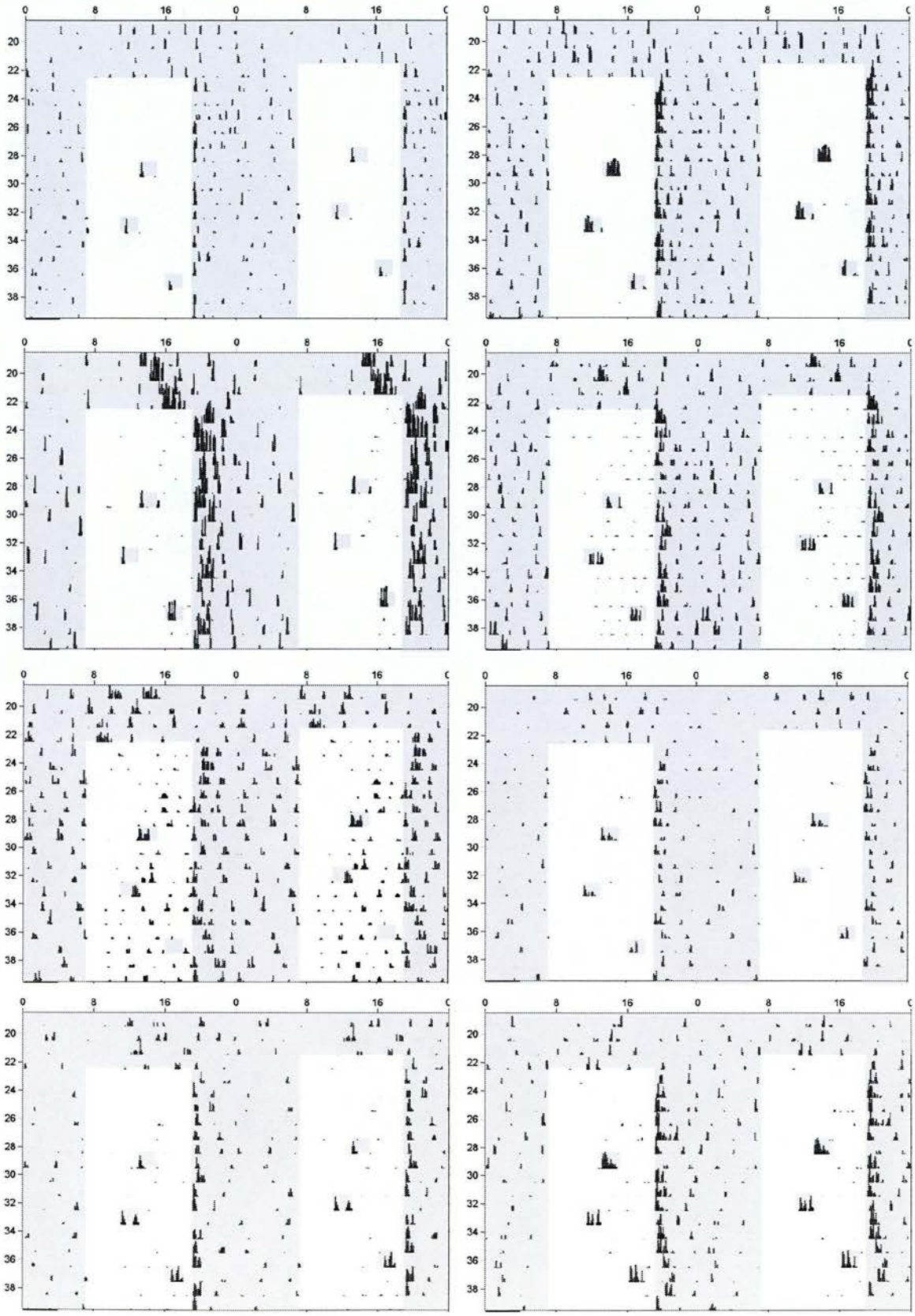
WT actograms Part 1a



WT actograms Part 1b



KO Dark pulse actograms Part 2a



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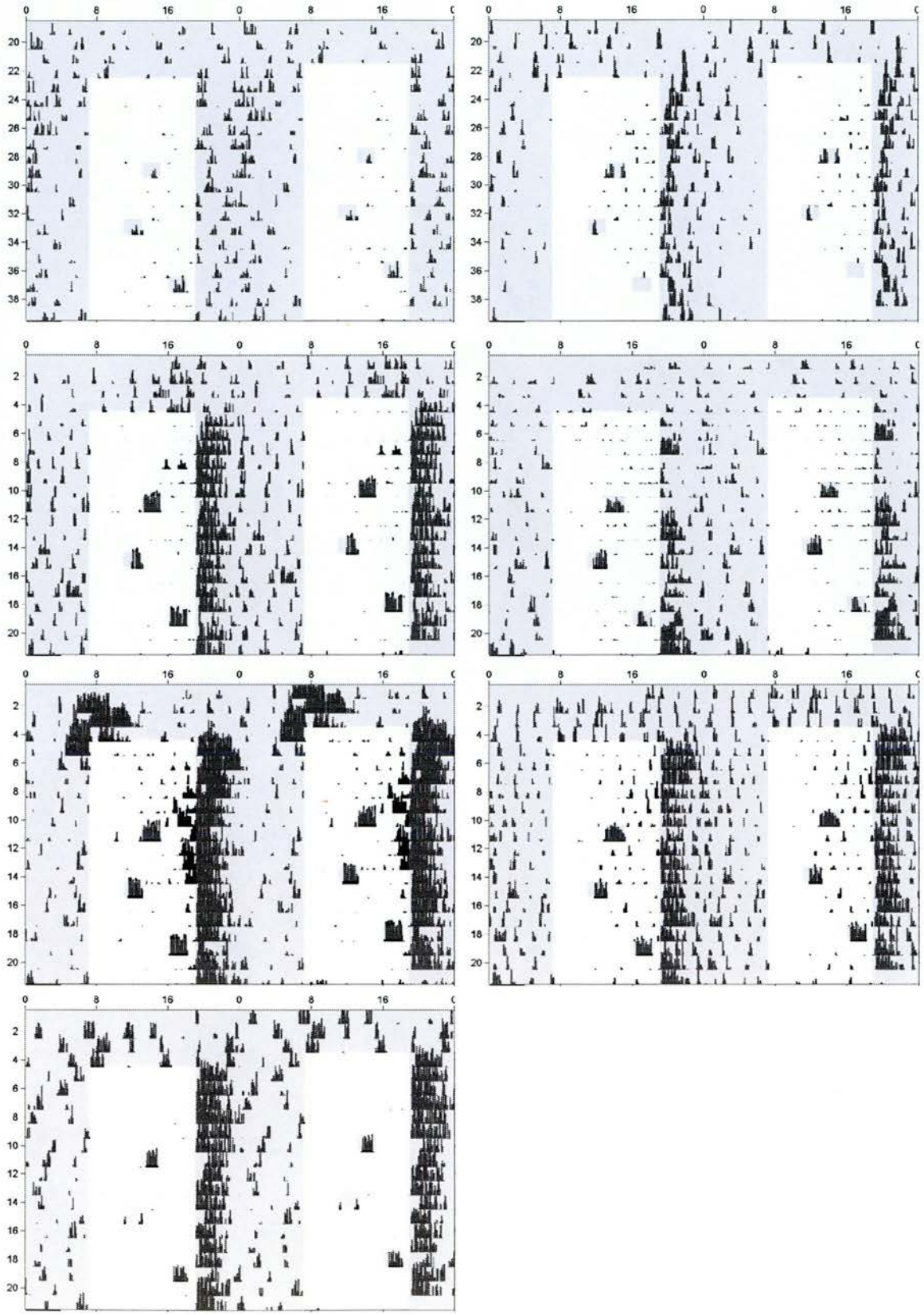
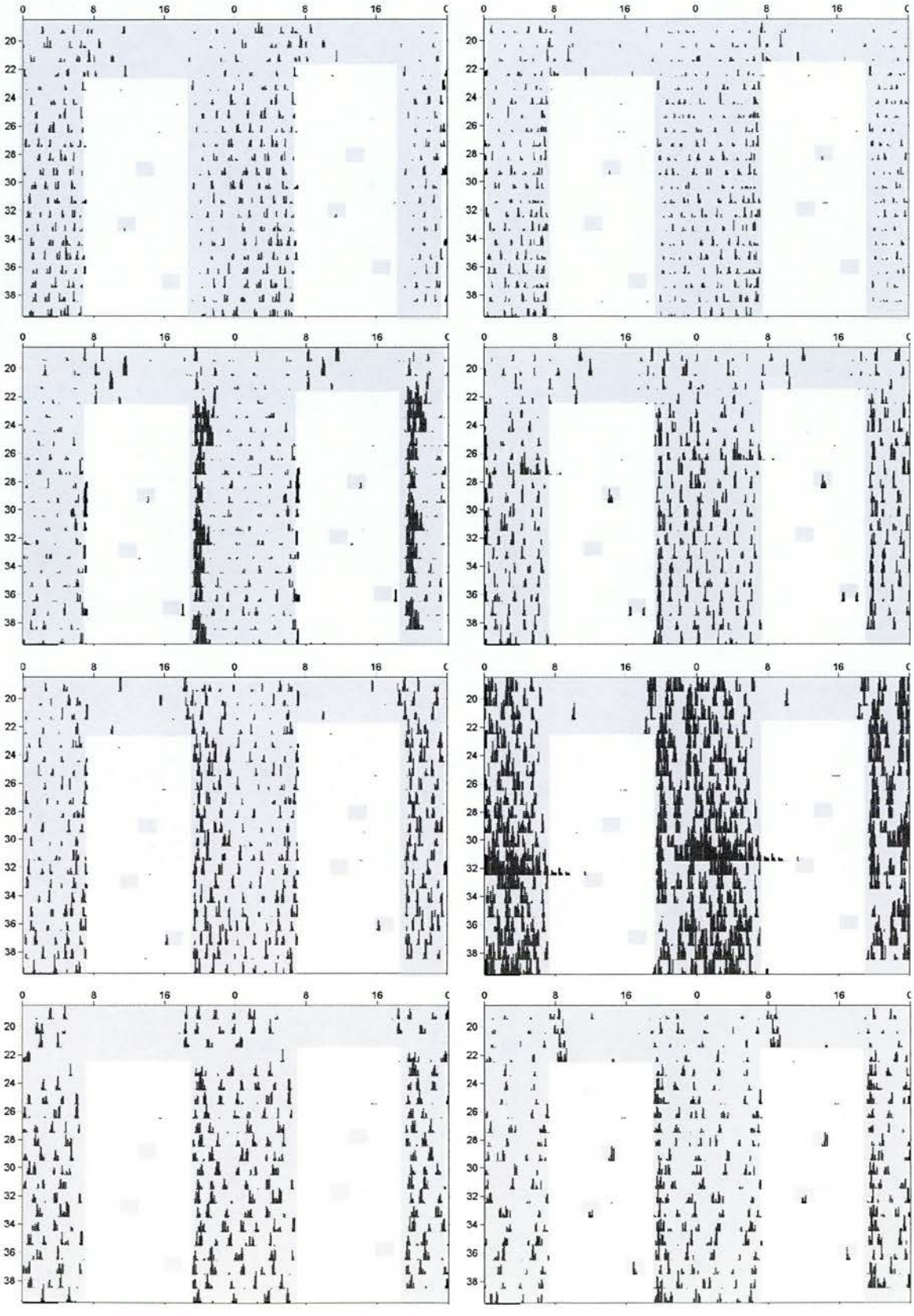
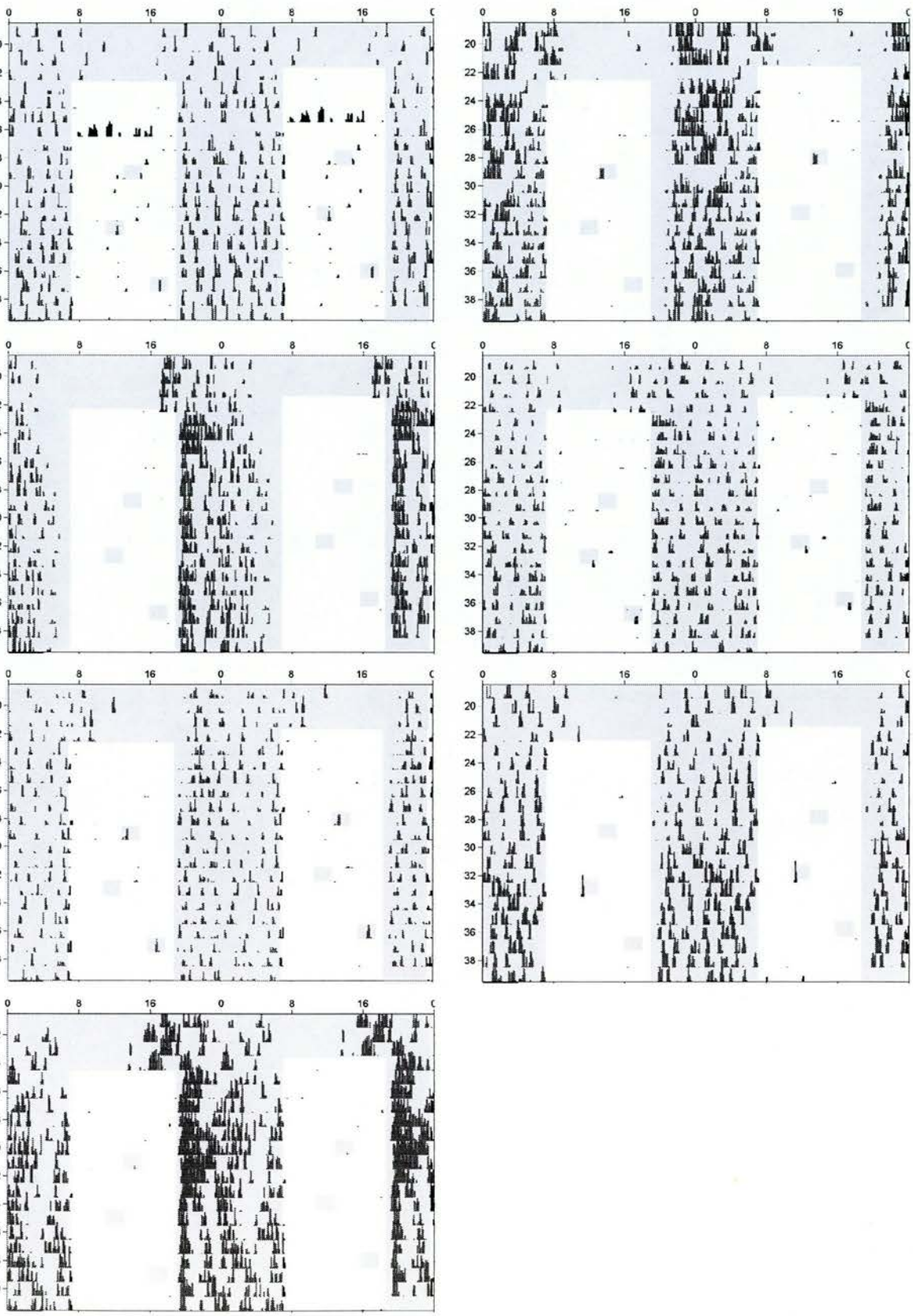


WT Dark pulse actograms Part 2a



WT Dark pulse actograms Part 2b



Appendix C

```
10 REM 9-Hole Box Signal Detection task (SDT) - V 4.00
20 REM Copyright Hugh H Marston and Christopher Spratt April 2001
40 REM use timer Z% to record intervals in box Z%
50 REM use timer 6 to record session length
60 REM use timer 7 to record session time
70 REM Uses hole 3 only, no contingencies on other responses
80 REM Dual intensity possibility
90 REM Includes stimulus duration by box
100 REM Print routines included
110 ver$="v4.00"
120 REM
130 REM ------------------------------------------------------------------------------
140 REM REFERENCE VARIABLES
150 hl%=0:      REM    operate House Lights
160 il%=1 :     REM    illuminate Hole 1
170 i2%=2:      REM    illuminate Hole 3
130 i3%=3:      REM    illuminate Hole 5
190 i4%=4:      REM    illuminate Hole 7
200 i5%=5:      REM    illuminate Hole 9
210 ml%=6:      REM    illuminate Magazine
220 or%=7:      REM    operate Reinforcer.
230 mr%=8:      REM    Magazine report
240 rl%=11:     REM report Hole 1
250 r2%=12:     REM report Hole 3
260 r3%=13:     REM report Hole 5
270 r4%=15:     REM report Hole 7
290 r5%=16      REM report Hole 9
290 nb%=3:      REM highest box ID number
300 Rep%=0:     REM Set Repeat Run Counter to Zero
310 filetype$="fff"
340 DIM sd%(nb%):       REM Stimulus Duration by Box
350 DIM si%(nb%):       REM Stimulus Intensity
360 DIM S%(nb%):        REM Random intensity variable
370 DIM Data%(nb%,4)
380 DIM NAME$(5)
390 session%=15*6000            : REM session length
400 iti%=500                    : REM inter trial delay
410 pe1%=20                     : REM pallet dispenser interval (sec)
420 REM ------------------------------------------------------------------------------
430 REM SET DIMENSIONS TO ZERO
440 FOR I%=0 TO nb%
450     sd%(I%)=0
460     si%(I%)=0
470     S%(I%)=0
490       FOR J%=0 TO 4
490             Data%(I%,J%)=0
520       NEXT J%
530 NEXT 1%
540 REM--------------------------------------------------------------------------
550 REM SETUP INPUTS AND OUTPUTS
560 PROCinit
570 PROCkill_all
530 REPEAT
590 FOR I%=0 TO nb%
600     FOR J=8TO 15:PROCfree_switch (J%+16*I%,E%): NEXT J%
610     FOR J%=0 TO 7:PROCgovn_switch (J%+16*I%,E%): NEXT J%
620 N E X T. 1%
630 C%=0
```

```
640 REM ------------------------------------------------------------------------
650
660 REM RUN PROGRAM
670
680 @%=&00005
690 CLS:PRINT"SIGNAL DETECTION PROGRAM          (SDT)"ver$'
691 INPUTTAB(5,3)"Treatment                              : "Treat$
701 INPUTTAB(5,5)"Session Length                        (Minutes): "session%
710 INPUTTAB(5,7)"Inter-Trial Interval                  (msec):  "iti%
720 INPUTTAB(5,9)"Limited hold                          (mSec): "lh%
730 INPUTTAB(5,11)"Time out duration                    (msec):  "to%
740 PROCdatafile
750 PRINTTAB(0,20) "Name mice in each test box "
760 FOR I%=O TO nb%:PRINTTAB(4,22+2*I%) "Box "I%+1": " :NEXTI%
770 FORI%=O TO nb%: INPUTTAB(17,22+2*I%)NAME$(I%):NEXTI%
780 PRINTTAB(29,20)"and stimulus duration (mSec)"
790 FORI%=O TO nb%:INPUTTAB(24,22+2*I%)sd%(I%):NEXTI%
800 CLS:PRINT"SIGNAL DETECTION PROGRAM          (SDT)"ver$'
801 PRINTTAB(5,3)"Treatment                          : "Treat$
810 PRINTTAB(5,5)"Session Length        (Minutes): "session%
820 PRINTTAB(5,7)"Inter-Trial Interval  (msec): "iti%
830 PRINTTAB(5,9)"Limited hold          (mSec): "lh%
840 PRINTTAB(5,11) "Time, out duration  (msec): "to%
850PRINTTAB(5,13)"TRIALS BY BOX"
860 FORI%=O TO nb%:PRINTTAB(20+8*I%,13)NAME$(I%):NEXTI%
870 PRINTTAB(5,15)"Stim Duration"
880 FORI%=O TO nb%:PRINTTAB(20+8*I%,15)sd%(I%):NEXTI%
890 PRINTTAB(8,17)'-Hit          Miss     CR       FA       "
900 REM PRINTTAB(5,17)"Responded Low"
910 REM PRINTTAB(5,19)"Rewarded High"
920 REM PRINTTAB(5,21)"Time out Low"
930 REM PRINTTAB(5,23)"Time out High"
940 session%=session%*6000
950 iti%=iti%/10
960 lh%=1h%/10
970 to%=to%/10
980 FORZ%=O TO nb%:sd%(Z%)=sd%(Z%)/10:NEXTZ%
990 PROCpipe_timer (6,session%,0,"FNendofsession (",0,E%)
1000 FOR Z%=0 TO nb%
1010     PROCswitch_off((hl%+16*Z%),E%)
1020     PR0Cpipe_timer(Z%,100,0,"FNreinforceon(",Z%,E%)
1030 NEXT Z%
1040 TIME=O:   REM Set Session Clock to Zero
1050 PROCpipe_timer (7,100,100, "FNscreen-clock ( ",0,E%)
1060 PROCwait (E%): *AE
1070 UNTIL Rep%<O
1080 END
1090 REM ------------------------------------------------------------------------
1100
1110 REM DEFINE FUNCTIONS AND PROCEDURES
1120
1130 DEFPROCdatafile
1140 INPUTTAB(5,16) "Datafile                                  :"datafile$
1150 IF datafile$=19"THEN
1160     PRINTTAB(45,16)"No Filename - Data will not be saved"
1170 ELSE
1180     XX%=OPENIN("$.OP.SRT-Data."+datafile$)
1190     IF XX%<>0 THEN
1200         INPUTTAB(45,16) "File Name Used Previously"K$
1210         CLOSE£ XX%
```

```
1220        PROCdatafile
1230    ELSE
1240        PRINTTAB(48,16)"File name 0K "datafile$
1250        CLOSE£ XX%
1260    ENDIF
1270 ENDIF
1280 XX%=OPENOUT("$.OP.SRT-Data."+datafile$)
1290 ENDPROC
1300
1310 DEFFNreinforceon(Z%,R%)
1320 IF R%=0 =0
1330 PR0Cpipe_switch(mr%+16*Z%,Over,l,"FNmag_resp(",Z%,E%)
1340 PROCswitch_on(m1%+16*Z%,E%)
1350 PROCswitch_on(or%+16*Z",E%)
1360 PR0Cpipe_timer(Z%.+10,pel%,0,"FNreinforceoff(",Z%,E%)
1370 =0
1380
1390 DEFFNreinforceoff(Z%,R%)
1400 IF R%=0 =0
1410 PROCswitch_off(or%+16*Z*%,E%)
1420 =0
1430
1440 DEFFNmag_resp(Z%,R%)
1450 IF R%=0 =0
1460 PROCkill_switch(mr%+16*Z%,EM
1470 PROCswitch_off(m1%+16*Z%,E%)
1480 PR0Cpipe_timer(Z%,iti%,0,"FNlight_hole(",Z%,E%)
1490 =0
1500
1510 DEFFNlight_hole(Z%,R%)
1520 IF R%=0 =0
1530 S%(Z%)=RND(2)
1540 REM l=DIM/NOISE 2=BRIGHT/SIGNAL
1550 PROCswitch_on((S%(Z%)+1)+16*Z%,E%)
1560 PR0Cpipe_timer(Z%,sd%(Z%),0,"FNcue_off(",Z%,E%)
1570 PR0Cpipe_timer(Z%+20,(sd%(Z%)+1h%),0,"FNno_response(",Z%,E%)
1580 PROCpipe_switch((i3%+10)+16*Z%,On,1,"FNnose_poke("..Z%,E%)
1590 =0
1600
1610 DEFFNcue off(Z%,R%)
1620 IF R%=0 =0
1630 PROCswitch_off((S%(Z%)+1)+16*Z%,E%)
1640 =0
1650
1660 DEFFNno_response(Z%,R%)
1670 IF R%=0 =0
1680 FOR I%=1 TO 5
1690    PROCkill.switch((I%+10)+16*Z%,E%)
1700    PROCswitch_off(I%+16*Z%,E%)
1710 NEXT I%
1720 PR0Cpipe_timer(Z%,iti%,0,"FNlight_hole(",Z%,E%)
1730 IF S%(Z%)=1 THEN
1740    Data%(Z%,3)=Data%(Z%,3)+1
1750    PRINTTAB(20,20+2*Z%)Data%(Z%,3)
1760 ELSE
1770    Data%(Z%,2)=Data%(Z%,2)+1
1780    PRINTTAB(14,20+2*Z)Data(Z%,2)
1790 ENDIF
1800 =0
1810
```

```
1820 DEFFNnose_poke(Z%,R%)
1830 IF R%=0 =0
1840 PROCkill_timer(Z%+20,E%)
1850 FOR I%=1 TO 5
1860              PROCkill_switch((I%+10)+16*Z%,E%)
1870              PROCswitch_off(I%+16*Z%,E%)
I880 NEXT I%
1890 IF S%(Z%)=2 THEN
1900              PR0Cpellet_on(Z%,R%)
1910    ELSE
1920    PROCnoise_resp(Z%,R%)
1930 ENDIF
1940 =0
1950
1960 DEFPROCnoise_resp(Z%,R%)
1970 PR0Cpipe_timer(Z%,to%+iti%,0,"FNlight_hole(".,Z%,E%)
1980 Data%(Z%,4)=Data%(Z%,4)+1
1990 PRINTTAB(26,20+2*Z%)Data%(Z%,4)
2000 ENDPROC
2010
2020 DEFPROCpellet_on(Z%,R%)
2030 PROCpipe_switch(mr%+16*Z%,Over,l,"FNmag_resp(",Z%,E%)
2040 PROCswitch_on(ml%+16*Z%,E%)
2050 PROCswitch_on(or%+16*Z%,E%)
2060 PR0Cpipe_timer(Z%+10,pel%,0,"FNreinforceoff(",Z%,E%)
2070 Data%(Z%,1)=Data%(Z%.1)+1
2080 PRINTTAB(8,20+2*Z%)Data%(Z%,1)
2090 ENDPROC
2100
2110 DEFFNendofsession(Z%,R%)
2120 IF R%=0 =0
2130 PROCzeroall
2.140 =0
2150
2160 DEFFNscreen__clock(Z%,R%)
2170 IF R%=0 =0
2180 PRINTTAB(2,40) TIME$
2190 T%=TIME :TMIN%=T%/6000:TSEC%=T%/100-60*TMIN%
2200 @%=3: PRINT TAB(66,9)"TIME :, "MIN%;: @%=2
2210 IF TSEC%<10 PRINT".0";TSEC%
2220 IF TSEC%>09 PRINT ".";TSEC%
2230 =0
2240
2250 DEFPROCzeroall
2260 PROCkill_all
2270 PROCdisc_save
2280 PROCnextsession
2290 ENDPROC
2300
2310 DEFPROCnextsession
2320 FOR I%=0 TO nb%
2330    sd%(I%)=0
2340    si%(I%)=0
2350    S%(I%)=0.
2360      FOR J%=0 TO 4
2370              Data(I%,J%)=0
2390    NEXT J%
2390 NEXT I%
2400 PRINTTAB(0,36)"END OF SESSION"
2410 PRINTTAB(0,38)"End of Testing (Y/n)  "
```

```
2420    CASE GET$ OF
2430            WHEN "Y","y" :Rep%=-1.
2440            WHEN "N","n": Rep%=1
2450    ENDCASE
2460 ENDPROC
2470
2480 REM ---- SAVE DATA TO DISC --------------------------
2490
2500 DEFPROCdisc_save
2510 IF datafile$="" THEN
2520    PRINTTAB(0,30)"END OF SESSION"
2530 ELSE
2540    PRINTTAB(0,30)"END OF SESSION : sending data to disc file "datafile$;
2550
5260    BPUT£ XX%, TIME$
2570    BPUT£ XX%, datafile$+","+ver$+","Treat$
2580    BPUT£ XX%, STR$ (session%* 10)+"+STR$ (iti%* 10) +","+STR$(1h%*10)+",
+ST$(to%*10)
2590    FOR I%=0 TO nb%
2600            BPUT£ XX%, NAME$(I%)+","+STR$((sd%(I%))*10)
2610            BPUT£ XX%, STR$(Data%(I%,1))+","+STR$(Data%(I%,2))+" , ";
2620            BPUT£ XX%, STR$(Data%(I%,3))+","+STR$(Data%(I%,4))
2630    NEXT I%
2640    BPUT£ XX%," "
2650    CLOSE£ XX%
2660    OSCLI ("Settype $.OP.SRT-Data."+datafile$+"      "+filetype$)
2670 ENDIF
2680 PROChard_copy
2690 ENDPROC
2700
2710 DEFPROChard_copy
2720 *FX5,1
2730 @%=6
2740 REM VDU2,1,27,1,15,3 : REM set condensed mode on EPSON printer
2750 CLS:VDU2: REM turn printer on and clear screen
2760 PRINT" **********          Signal Detection Program (SDT) "ver$;
2770 PRINT" ********** "
2780 PRINT ''
2781 PRINTTAB(5)"Treatment                              : "Treat$
2790 PRINTTAB(5)"Session Length      (Min)   : "session%/6000
2800 PRINTTAB(5)"Inter-Trial Interval   (Sec)   : "iti%/100
2810 PRINTTAB(5)"Limited hold        (Sec)   : 1h%/100
2820 PRINTTAB(5)"Time out duration   (Sec)   : "to%/100
2930 PRINT ''
2840 PRINT"Mouse     Hit     Miss    CR      FA      Stim D "
2850 FOR I%=0 TO nb%
2860    PRINT NAME$(I%),;
2870    FOR J%=1 TO 4
2880            PRINT Data%(I%,J%),;
2890    NEXT J%
2900 PRINT sd%(I%);
2910 PRINT '
2920 NEXT I%
2930 PRINT'
2940
2950 @%=5:PRINT '''
2960 PRINT TIME$
2970 CLS
2980 VDU3
2990 ENDPROC
```